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Functional studies of genetic variants of unknown significance

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Mestrado em Biologia Molecular e Genética

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2019

AGRADECIMENTOS

Este último percurso académico, que culminou na elaboração deste trabalho não teria sido possível sem o empenho e estímulo de diversas pessoas. Gostaria de expressar toda a minha gratidão e apreço a todos aqueles que, direta ou indiretamente, contribuíram para que esta tarefa se tornasse uma realidade, e que influenciaram a minha viagem académica e crescimento a nível pessoal. A todos quero manifestar os meus sinceros agradecimentos.

Em primeiro lugar, um especial agradecimento à minha orientadora Professora Doutora Susana Nunes da Silva, para quem não há agradecimentos que cheguem e que foi mais do que uma orientadora para mim. Por todo o apoio e ensinamentos ao longo deste ano, pela liberdade de ação que me permitiu, de forma decisiva para que este trabalho contribuísse para o meu desenvolvimento pessoal.

Ao Professor Sebastião Rodrigues, o modo como se disponibilizou no auxílio dos trabalhos e pela partilha do seu conhecimento e experiência.

Ao Professor José Rueff, por me receber no ToxOmics e pela amabilidade e gentileza que sempre demonstrou, pelo interesse, preocupação e disponibilidade por ajudar e partilhar o seu conhecimento e experiência.

Às minhas companheiras de laboratório, principalmente à Maria João Pires e à Mónica Honrado por se terem tornado grandes amigas durante este ano e por toda a ajuda que disponibilizaram tanto a nível de elaboração deste trabalho, como a nível pessoal.

À Cláudia Andrade por toda a ajuda e disponibilidade no manuseamento da aparelhagem da citometria de fluxo.

Às meninas da nutrição por toda a conversa e alegria que transmitiam.

Ao grupo do laboratório 2.16, mais concretamente Bruno Gomes, Francisco Esteves e Marisa Cardoso, que sempre se disponibilizou para ajudar no trabalho e me apoiar incansavelmente a nível pessoal.

À Rita Lourenço pela paciência e partilha de ensinamentos e experiências que transmitiu na execução deste trabalho.

Aos meus amigos que nunca estiveram ausentes, agradeço a amizade e carinho que sempre me disponibilizaram.

Por último, e sem dúvida mais importante, um eterno agradecimento à minha família pelo apoio incondicional que me deram toda a vida, que sempre acreditou no meu potencial e que sem eles ao meu lado não teria conseguido atingir os meus objetivos. Um especial agradecimento aos meus pais e avós por sempre demonstrarem orgulho e carinho por mim e por acreditarem sempre em mim, nos bons e maus momentos da vida, e que sem eles eu não estaria onde estou hoje. À minha querida irmã Margarida, que mais do que uma irmã, é uma amiga para todas as ocasiões da vida.

A todos, muito obrigado por permitirem que esta tese seja uma realidade.

FUNDING

This study has been supported by funding through a Terry Fox 2017 grant from Liga Portuguesa Contra o Cancro (LPCC).

ABSTRACT

Breast cancer is the second most commonly diagnosed cancer worldwide and the most common cancer among women. It is the family history or genetic predisposition the most important factor of breast cancer risk. In fact, 5-10% of cancer cases are hereditary breast cancer (HBC), and mainly of them are associated with inherited mutations in *BRCA1* and *BRCA2* genes. These genes are involved in both the main DNA DSB repair pathways - non-homologous end-joining (NHEJ) and, the most important, homologous recombination (HR). So, mutations in these genes could cause defectives in DNA repair, and because of that, cells that suffer serious unrepaired damage might triggers the development of breast cancer. To avoid that, it is very important to perform clinically a genetic testing, which could identify individuals and families with genetic predisposition to developing cancer, like mutations in *BRCA1* and *BRCA2*. Unfortunately, in 12%-13% of the patients tested for *BRCA1/2* mutations, are detected VUS, or variants of uncertain significance, which does not know if the variant is either benign or pathogenic, which generates significant problems in risk evaluation, counselling and preventive care. To overcome this problem, several functional assays have been or are being developed to clarify its mutational status. Our goal with this study is a functional evaluation of the role of VUS previously identified, in drug response (Doxorubicin). To doing so, we performed a proof of concept functional analysis using Peripheral Blood Mononuclear Cells (PBMCs) collected by venous puncture from four Portuguese volunteers: two VUS-carriers patients in the *BRCA1* gene and two negative controls. In this study we selected 4 techniques to assess the cellular response to lesions induced by Doxorubicin: TUNEL assay, Caspases activity assay, γH2Ax assay and Comet assay.

The results from TUNEL assay and Caspases activity assay had some differences between each other, mainly due the existence of intra-individual variability, what makes essential to perform another's assays. The results for γH2Ax and Comet assays besides showing intra- and inter-individual variabilities, showed an increase of sensitivity possibly associated with a defective in DNA repair for VUS-carriers, after exposure to increased doses of doxorubicin. In fact, except TUNEL assay, all techniques show this sensitivity when the samples were treated with Doxorubicin. However, and mainly because of the existence of genetic variability additional assays and studies should be performed to understand the results obtained and to possibly characterize the highly frequent VUS in cancer-related genes and give us some answers about breast cancer risk.

Keywords: Breast cancer; *BRCA1*; DNA repair pathways; VUS; PBMCs; Functional assays

RESUMO

Mundialmente a segunda causa mais comum de morte pertence ao cancro, um grupo de doenças relacionada com uma grande instabilidade genómica e que leva a um crescimento descontrolado de células anormais. Incluído na vasta gama de cancros, o cancro da mama está no segundo lugar de cancros mais comuns diagnosticados globalmente, sendo ainda o mais comum entre as mulheres. Dentro de diversos fatores como estilo de vida, dieta, intervenções médicas de longo prazo, sexo e etnia da pessoa e base genética, o fator mais importante que eleva o risco de cancro da mama pertence à história familiar ou predisposição genética. De facto, 5%-10% de casos de cancro da mama são hereditários (HBC) e a maior parte deles estão associados com mutações herdadas nos genes *BRCA1* e *BRCA2*. Estes genes estão envolvidos em ambas as vias de reparação de quebras de dupla cadeia do DNA – união de extremidades não-homólogas (NHEJ) e, a mais importante, recombinação homóloga (HR). Logo, mutações nestes genes, que contribuem para 20%-25% de risco, podem causar deficiência na reparação de DNA, e com isso, células que sofrem sérias lesões, ao não serem reparadas, potencia o aparecimento de cancro da mama. Essas lesões têm diversas origens, que podem ir desde processos metabólicos, como espécies reativas de oxigénio (ROS), até fatores externos como radiação ou agentes químicos. Para evitar isso, é muito importante realizar clinicamente testes genéticos, que podem identificar individualmente ou numa família uma predisposição genética para desenvolver cancro, como mutações em *BRCA1* e *BRCA2*. Hoje em dia já existe tecnologia que permite uma sequenciação mais completa do genoma e que permite fornecer informações a respeito de alteração de regiões de codificação que poderão estar associadas ao cancro. Apesar dos custos desta tecnologia, sequenciação de próxima geração (NGS), serem ainda altos, uma sequenciação de genes baseada nela é melhor escolha para o diagnóstico de alguns pacientes. Contudo, e apesar desta enorme inovação tecnológica, em cerca de 12%-13% dos pacientes testados para mutações *BRCA1/2* são detetadas VUS, ou variantes de significado desconhecido, maioritariamente alterações missense, e que portanto, não se sabe se a variante é benigna ou patogénica, o que acaba por gerar problemas significativos na avaliação do risco, aconselhamento e cuidado preventivo. Para ultrapassar este problema, diversos ensaios funcionais estão a ser ou vão ser desenvolvidos para clarificar o estado mutacional da variante e obter dados que possam prever a casualidade da VUS num modelo teórico. Por esse mesmo motivo, e sabendo que a divisão celular pode ser tida como alvo para tratar pacientes com cancro, é muito útil o uso de agentes quimioterapêuticos na construção destes ensaios funcionais. Estes agentes induzem lesões no DNA que são reconhecidas pelos sistemas de reparação e levam à reparação ou morte das células, no entanto de referir, que sendo agentes quimioterapêuticos, apenas causam dano no DNA em altas doses. Contudo, tal como o nome sugere, nas VUS, é totalmente desconhecido se os mecanismos de reparação de DNA são interrompidos aquando de mutações nos genes responsáveis pela sua reparação, como *BRCA1* e *BRCA2*, que por sua vez estão ligados a cancro da mama e ovários. Por esse motivo, portadores de VUS diagnosticados com cancro metastático da mama ou ovário podem não beneficiar de certas terapias, como inibidores de platina ou PARP, que têm como alvo os defeitos do sistema de reparação de DNA, e que são atualmente recomendados para indivíduos com variantes patogénicas de *BRCA1* ou *BRCA2*. Daí ser tão importante a realização de trabalhos, como este mesmo estudo que permite uma avaliação funcional do papel das VUS, previamente identificadas, em resposta a agentes genotóxicos (Doxorubicina).

Para possibilitar a execução de um estudo funcional a estas VUS previamente sequenciadas, realizámos uma "prova de conceito" de análise funcional em Células Mononucleares de Sangue Periférico (PBMCs) coletadas por injeção venosa e derivadas de quatro voluntárias portuguesas com um diferente diagnóstico para cancro da mama hereditário: dois portadores de VUS para o gene *BRCA1 e* dois controlos negativos. Neste estudo selecionámos quatro técnicas para avaliar a resposta celular a

lesões induzidas por Doxorubicina: Ensaio de TUNEL, ensaio de atividade de Caspases, ensaio de γH2Ax e ensaio do "cometa". O ensaio de TUNEL é um método de deteção da fragmentação de DNA apoptótico *in situ*, que como é sugerido, identifica tanto células em apoptose, como lesões no DNA, quando usado isoladamente. O ensaio de atividade das Caspases permite a deteção de caspases ativas, ou seja, de proteases cisteínicas intracelulares e que desempenham papéis fundamentais na via da apoptose e inflamação. Desta forma, com este método, ao detetar caspases que estejam ativas estamos a detetar diferentes fases de apoptose das células. Em relação à metodologia de γH2Ax, é um método que se baseia na análise de marcadores para quebras de dupla cadeia do DNA (DSBs). Estes marcadores internos, alteram a sua conformação quando o DNA sofre lesões e por esse motivo, ao quantificar esses marcadores alterados, é possível quantificar as células afetadas por determinado estímulo. Por último, o método do "cometa" possibilita a quantificação de células que sofreram dano no DNA por meio de análise da quantidade de DNA que migrou para fora do núcleo quando a mesma célula sofreu um determinado estímulo. É a esta migração de DNA que se dá o nome de cometa, visto apresentar uma aparência semelhante. Todas estas técnicas, com exceção do método do "cometa" que é analisada microscopicamente, são realizadas em citometria de fluxo, uma vez que se trata de uma metodologia altamente sensível para avaliação dos resultados.

Os resultados obtidos provenientes do TUNEL e da atividade de Caspases tiveram algumas diferenças entre si, muito devido a variabilidade intra- e inter-individual, o que torna essencial realizar novos ensaios. Relativamente ao γH2Ax e ao ensaio do cometa, os resultados para além de mostrarem as referidas variabilidades, intra- e inter-individuais, também mostra um aumento de sensibilidade possivelmente associada com uma deficiência na reparação de DNA com o aumento de dose de Doxorubicina, para as amostras de portadores de VUS. De facto, exceto o TUNEL, todas as técnicas mostram essa sensibilidade quando as amostras são tratadas com o agente. No que toca aos controlos positivos (Camptotecina - CPT e Peróxido de Hidrogénio - H_2O_2), os resultados não são os mais esperados, contudo enquanto nos estudos de apoptose, o CPT mostra níveis de apoptose, mesmo que poucos, nos estudos de quantificação de lesão no DNA, a H_2O_2 mostrou danos elevados em apenas algumas amostras, levando a crer novamente a existência de variabilidade inter-individual neste estudo. Deste modo, em ensaios futuros é essencial testar a eficiência destes mesmos controlos para um melhor controlo e entendimento dos resultados obtidos. Para além disto tudo, estes resultados são o exemplo da grande limitação que o uso de células mononucleares de sangue periférico (PBMCs) oferecem a estudos deste género, visto que as colheitas intensivas de sangue por injeção são de certo modo, invasivas quer para os voluntários, limitando por uma questão ética a obtenção de células a serem usadas nos trabalhos. Desta forma, um dos nossos objetivos futuros é o estabelecimento de uma linha imortalizada de linfócitos B humanos que poderá ajudar a ultrapassar este problema. Contudo, enquanto as linhas não estão prontas e, em grande parte devido à existência de variabilidade genética, devem ser realizados mais ensaios e estudos, de forma a compreender melhor os resultados obtidos e para ser possível caracterizar as VUS mais frequentes em genes relacionados com cancro e nos dar algumas respostas sobre o risco de cancro da mama.

Palavras-chave: Cancro da mama; *BRCA1*; Vias de reparação de DNA; VUS; PBMCs; Ensaios funcionais

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LIST OF ABBREVIATIONS, GENES, PROTEINS AND CHEMICAL COMPOUNDS

% DNA in Tail	Percentage of DNA in Tail
γH2Ax (%)	Percentage of DNA in Tail
ANOVA	Analysis of variance
Apototic rate (%)	Percentage of apotosis
BC	Breast Cancer
BER	Base-excision repair
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
BrdUTP	5-Bromo-2´-Deoxyuridine 5´-Triphosphate
BSA	Bovine serum albumin
CEFCM	Ethical Commission of NMS/FCM
Cell population (%)	Percentage of cells
CPT	Camptothecin
CRIPSR	Clustered Regularly Interspaced Short Palindromic Repeats
DDR	DNA damage response
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
DSB	Double strand break
DSBR	Double-strand DNA break repair
EDTA	Ethylenediamine tetraacetic acid
FBS	Fetal Serum Bovine
FLICA	Fluorochrome Inhibitors of Caspases
FSC	Forward scatter
H2Ax	Histone H2Ax
H_2O_2	Hydrogen peroxide
HBC	Hereditary breast cancer
HR	Homologous recombination
IR	Ionizing Radiation
MMEJ	Microhomology-mediated end-joining
MMR	Mismatch repair
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NC	Negative Control
Necrotic rate (%)	Percentage of necrosis
NER	Nucleotide-excision repair
NGS	Next generation sequencing
NHEJ	Non-homologous end-joining
NMS/FCM	Nova Medical School
PARP	Poly (ADP-ribose) polymerase
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
Pen-Strep	Penicillin-Streptomycin
PI	Propidium Iodide
RNA	Ribonucleic Acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCGE	Single cell gel electrophoresis
SD	Standard deviation
SSB	Single strand break
SSC	Side scatter
TdT	Terminal deoxynucleotidyl transferase
Tris Base	Tris(hydroxymethyl)-aminomethane
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick- End Labeling
TUNEL-positive cells (%)	Percentage of apototic cells
UV	Ultraviolet
VUS	Variants of unknown significance

1. INTRODUCTION

1.1 CANCERIGENESIS

Cancer is a generic term for a large group of diseases, which it is usually characterized as an evolutionary process that results from the accumulation of somatic mutations in normal cells, leading to an uncontrolled growth/proliferation of the mutated cells that can invade adjoining parts of the body and/or spread to other organs (Blanpain, 2013; WHO, 2014). It is the second leading cause of death worldwide and is estimated to account for 9.6 million death in 2018. The most common types of cancer in men are lung, prostate, colorectal, stomach and liver cancer, while in women, the most common are breast, colorectal, lung, cervix and thyroid cancer (Hassanpour & Dehghani, 2017; WHO, 2014).

Biologic molecules are very sensitive to spontaneous chemical reactions, mostly hydrolysis, and DNA is the most vulnerable, because it cannot simply be replaced, instead of it, it relies on repair of existing molecules. It is represented by only one copy in most cells and accumulates numerous lesions over a lifetime. The bases in DNA are highly sensitive to chemical modifications, which can cause serious damage to DNA molecule. When defective repair and replication errors convert these damages into mutations, the changes are permanent and continually exercise their effect, even in successor cells. Furthermore, these mutations cause the loss of tumor-suppressor genes and the activation of oncogenes, which trigger uncontrolled cellular proliferation and the development of abnormal cells (Hoeijmakers, 2009; Roos & Kaina, 2006).

The resultant DNA damage either involve one (single strand break – SSB) or both strands of the DNA (double strand break – DSB), which occurs when the sugar-phosphate backbones of both DNA strands are broken at a similar position or near one other. While SSBs can be converted to DSBs and be repaired, unrepaired DSBs can lead to serious and permanent consequences in cells, once they can be mutagenic and affect the expression of multiple genes, and most importantly, be lethal to the cell (Nowsheen & Yang, 2013).

1.2 DNA DAMAGING AGENTS

DNA can be damaged by products from exogenous processes, such as those from external environmental processes, and endogenous processes, from internal metabolic processes. Endogenous agents include: reactive oxygen and nitrogen species (ROS and RNS, respectively), formed during oxidative stress, metabolic processes and the inflammatory response; depurination and depyrimidination at certain loci, which occurs through the hydrolysis of N-glyosidic bonds; and replication stress, which occurs during the S phase and causes the stalling of replication forks. On the other turn, exogenous agents include: ionizing and solar ultraviolet radiation, which generates, respectively, SSBs and DSBs as well as oxidative modifications of nucleobases, and also pyrimidine dimers, which distort the structure of the DNA chain and blocks transcription or replication past the site of damage; environmental pollutants present in air, water and food; and chemical agents (second-hand smoke, pesticides and toxic metals), which are metabolised into highly reactive metabolites that react with nitrogenous bases and can lead to deleterious DNA strand breaks and DNA adducts (Copper, Geoffrey and Hausman, 2007; Helena et al., 2018; Hoeijmakers, 2009; Nowsheen & Yang, 2013).

To maintain the genomic integrity, cells developed two strategies: DNA damage is repaired or tolerated, by DNA damage response (DDR), after detection the above lesions; or cells are removed by death, if they possess DNA damage too great or when the repair is ineffective (Nowsheen & Yang, 2013; Roos & Kaina, 2006). Basically, after DNA damage, sensor proteins bind to and signal to cell cycle checkpoint and DNA damage checkpoint kinases, which will induce cell cycle arrest and start the appropriate DNA damage repair pathway to deal with the type of damage present. After that, if the repair was successful, the cell survives and resumes replication, if not, cell death or senescence pathways are triggered. If the DNA repair mechanisms are defective genomic instability arises, which is one of the hallmarks of cancer (Figure 1.1) (Majidinia & Yousefi, 2017).

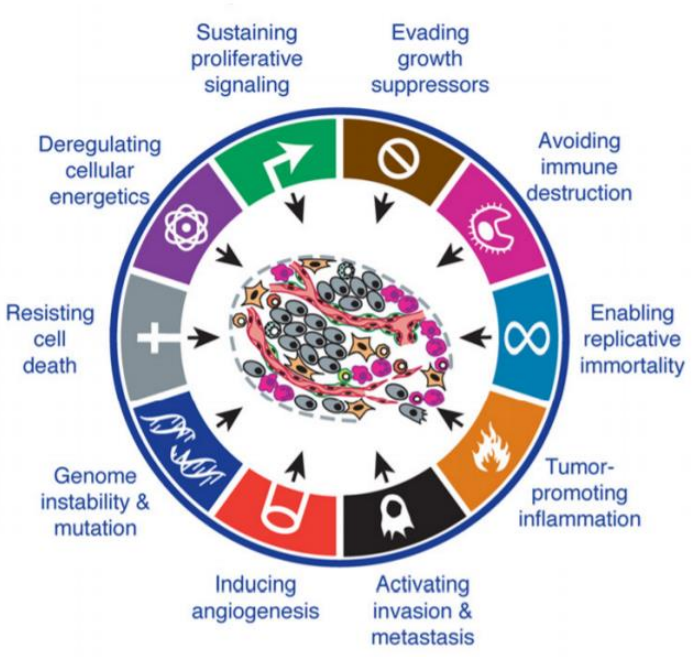

Figure 1.1 - Hallmarks of Cancer (Hanahan & Weinberg, 2011)

1.3 DNA REPAIR

The prominent DNA repair mechanisms (Figure 1.2) include mismatch repair (MMR), base-excision repair (BER), nucleotide-excision repair (NER), and double-strand DNA break repair (DSBR), which can be further divided in non-homologous end-joining (NHEJ), homologous recombination (HR), and microhomology-mediated end-joining or alternative end-joining (MMEJ), which is an alternate NHEJ pathway that commonly results in DNA sequence deletions (Helena et al., 2018).

Mismatch repair controls the correction of base pair mismatches, which occur when A-G and C-T do not pair correctly and, also, corrects DNA insertions and deletions resulting from erroneous DNA replication or DNA polymerase misincorporation errors (Helena et al., 2018).

Base-excision repair (BER) is involved in the removal and replacement of damaged DNA bases, resulting from ROS, X-rays, alkylating agents and spontaneous reactions (Helena et al., 2018).

Nucleotide-excision repair (NER) controls the removal of DNA adducts from DNA by excising an oligonucleotide containing the lesion to replace it with newly synthesised DNA, which can be induced by UV light and polycyclic aromatic hydrocarbons which contribute to destabilization of the DNA double helix (Helena et al., 2018).

As mentioned before, the agents that cause DSBs include X-rays, ionizing radiation and anti-cancer drugs. Briefly, after these breaks been detected some members of the DDR machinery promote the formation of foci at sites of DNA damage leading to the recruitment of repair proteins. There are two main mechanisms occurring in the DSB repair process, NHEJ and HR. In NHEJ, no sequence homology for DSBs end-joining is required, and it involves minimal DNA processing, but when it occurs bases may be lost or added, and because of that it is associated with an elevated risk of mutagenesis. On the contrary, in HR, sequence homology is required in order to align DSBs ends prior to ligation, and because of that HR is the most important error-free DNA DSB repair mechanism. Briefly, in HR, replicated sister chromatid DNA sequences are used as templates to restore missing DNA sequences on the damaged chromatid, and because of that, it can only operate in the S and G2 phases of the cell cycle (Helena et al., 2018; Roos & Kaina, 2006).

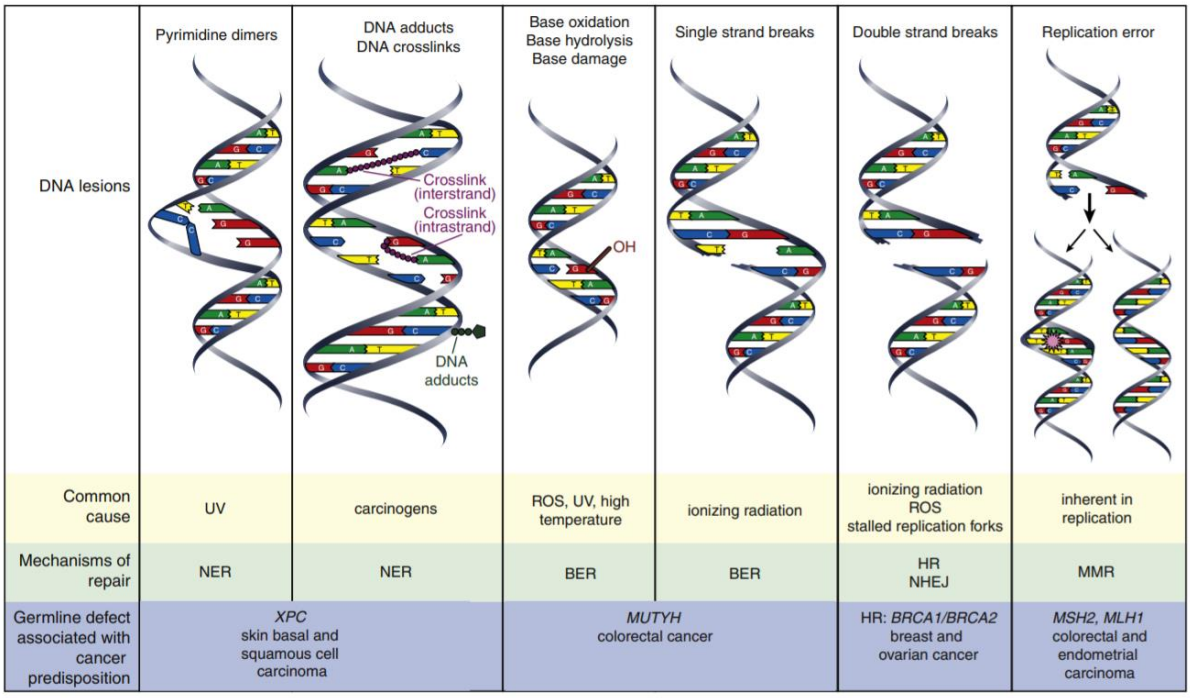

Figure 1.2 - Different types of DNA lesions, their origins, repair pathways and predisposing germline mutations leading to cancer. BER, base excision repair; IR, ionizing irradiation; HR, homologous recombination; MMR, mismatch repair; NER, nucleotide excision repair; NHEJ, non-homologous end joining; ROS, reactive oxygen species; UV, ultraviolet. (Weeden & Asselin-Labat, 2018)

So whatever is the type of DNA damage, the response to this genomic instability involves signalling, checkpoints activation, and repair of the DNA lesions, and therefore the maintenance system determines cell's fate, as mentioned before – survival, replicative senescence, or death, which can occur by apoptosis, necrosis or autophagy (Hoeijmakers, 2009; Nowsheen & Yang, 2013; Roos & Kaina, 2006). Programmed cell death or apoptosis is a physiological mechanism, involving specific morphological and biochemical changes such as cell shrinkage, chromatin condensation, protein cleavage, DNA breakdown and phagocytosis, while necrosis is a form of cell death that causes cytoplasmic swelling, dilution of cytoplasmic organelles, rupture of plasma membrane and some chromatin condensation. On the other hand, autophagy occurs without chromatin condensation, and forms two-membrane autophagic vacuoles that contain degenerating organelles or cytosol (Papaliagkas, Anogianaki, Anogianakis, & Ilonidis, 2007; Roos & Kaina, 2006; Ward et al., 2008).

1.4 BREAST CANCER

Breast cancer (BC) is a disease in which certain cells from the breast grow and divide uncontrollably, which subsequently turn into breast tumor. It is the second most commonly diagnosed cancer worldwide and the most common cancer among women, with an estimated 2.1 million cases and 626 679 deaths in 2018 (Lam & Ervik, 2018; Psyrri, Apostolou, Fostira, Economopoulou, & Dimitriadis, 2015). Although BC is much more common in women, it can also develop in men. Its development is associated with genetic and environmental factors, or risk factors, which it is defined as factors or agents that affects individual's chance of getting a specific disease, in this case breast cancer. As mentioned before, they can be divided into two groups: (a) inherent or genetic factors, such as gender, age, race and ethnicity and genetic background; and (b) extrinsic or environmental factors, such as long-term medical intervention, dietary habits and other factors conditioned by lifestyle (Feng et al., 2018; Kamińska, Ciszewski, Łopacka-Szatan, Miotła, & Starosławska, 2015). However, family history or genetic predisposition is the most important factor for determining breast cancer risk (de Jong et al., 2002). Actually, it is the genetic heterogeneity that is passed down under a autosomal dominant inheritance and that can be recognized clinically, early onset, by dominant inheritance (Mahdavi et al., 2019).

The definition of "familial" breast cancer is not simple, but it is generally accepted a criteria that includes: (a) at least three breast and/or ovarian cancer cases in a family; (b) two breast cancer cases in close relatives, with at least one case diagnosed before 50 years of age; (c) at least two breast cancer cases in a family diagnosed before age 40; (d) any male breast cancer case with a family history of ovarian cancer or early start female breast cancer; (e) Ashkenazi Jewish ancestry with breast cancer, especially triple-negative breast cancer and diagnosed before 60 years-old; and (f) breast and ovarian cancer in the same person (Shiovitz & Korde, 2015). The hereditary breast cancer (HBC) accounts for 5-10% of cancer cases, while the majority of breast cancers belong to sporadic subtype, which it is associated with an increasing accumulation of unrepaired mutations in breast cells that are acquired during patients life (Feng et al., 2018; Majidinia & Yousefi, 2017).

1.5 BREAST CANCER AND GENETIC PENETRANCE

In HBC, the genetic starting point is based on a hereditary germline mutation that occurs in one of the alleles from genes with highly penetrance, followed by a reduction in heterozygosity of somatic tissues. The high penetrance genes *BRCA1* and *BRCA2* account for 20-25% of this risk, almost one-third of the inherited BCs. Its proteins take a major part in repairing DSB which can be done by HR. Thus, BRCA defective cells are highly susceptible to DNA damage and, consequently, carcinogenesis (Mahdavi et al., 2019). In addition to the high penetrance genes, both moderate-penetrance genes, additional rare, and low-penetrance alleles, more common, are also associated with hereditary breast cancer (Figure 1.3) (Shiovitz & Korde, 2015).

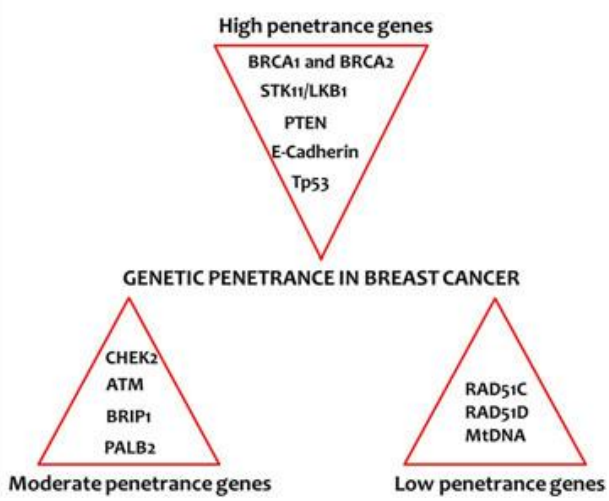

Figure 1.3 - Breast cancer and genetic penetrance: List of genes more frequently associated with high, moderate and low penetrance (Mahdavi et al., 2019)

1.6 GENETIC TESTING

There are a variety of reasons to perform clinically a genetic testing. They are divided in: (i) diagnostic testing, which is done to determine whether a patient with symptoms has a determined condition, or what condition they have, if their symptoms are non-specific; (ii) predictive testing or genetic risk assessment, which is done usually for people with a family history of a condition for which they have not yet presented symptoms, and can lead to clinical interventions, or be useful in life planning; and (iii) reproductive testing, which informs parents of the risk of having a child affected with a recessive genetic disorder, and determines whether a pregnancy is affected by a genetic condition (Hoffman-Andrews, 2017).

The major purpose of genetic testing is the identification of individuals and families with a genetic predisposition to developing cancer, like the identification of tumor suppressor genes associated with specific hereditary cancer syndromes, such as *BRCA1* and *BRCA2* (Carvalho, Couch, & Monteiro, 2007; Grogan & Kirsch, 1997). This is particularly important to educate at-risk family members about their risk and to promote changes in their life style, foster increased surveillance and to allow informed decisions about preventive surgery or hormonal therapy (Carvalho et al., 2007; Grogan & Kirsch, 1997). Unfortunately, there are limitations on genetic testing, such as the many complexities involved in its application, interpretation, clinical significance, and psychological effects to the patients and the many technical limitations that often result in limited sensitivity or an inability to detect all mutations in the gene of interest (Carvalho et al., 2007; Grogan & Kirsch, 1997). Nowadays, the use of next generation sequencing (NGS) for platforms and projects has provided comprehensive gene alteration data in protein-coding regions for all types of human cancer. In fact, NGS based genomic sequencing has enabled the sequencing of individual cancer-patient genomes, and it has become, year by year, faster and less expensive. However, when sequencing the whole exome (the coding part) they provide us more information than what can be practically analysed and interpreted at a level required for clinical application and it is too costly for individual patient diagnosis (Nagahashi et al., 2019). Still, when putting the both dishes on the scale, the use of genetic testing with NGS technology is of clearly more vital and important for the population, mainly to individuals with a family history of breast and/or ovarian cancer, once the NGS-based gene panel test is the first choice for individual cancer-patient care (Nagahashi et al., 2019).

The results from a *BRCA* genetic test generally fall into three categories: (a) Positive results, where a pathogenic mutation is found and it provides a diagnosis or risk information; (b) Negative results, where no relevant genetic alteration, mutation or polymorphism, is found; or (c) VUS, or variants of uncertain significance, where is found a genetic alteration, but the cancer risk has not been determined (Hoffman-Andrews, 2017; Mahdavi et al., 2019).

1.7 VARIANTS OF UNCERTAIN SIGNIFICANCE (VUS)

VUS, detected in 12-13% of patients tested for *BRCA1* and *BRCA2* mutation status, are variants of unknown clinical impact, and include mostly missense variants (that lead to a single amino acids change), but also small in-frame deletions or insertions, synonymous nucleotide substitutions, certain truncating mutations (such as mutations in the last exons of genes), as well as alterations in noncoding sequences or in untranslated regions (Mahdavi et al., 2019; Moghadasi, Eccles, Devilee, Vreeswijk, & van Asperen, 2016). Some of these variants behave as low-penetrance gene mutations and should not be treated in same way as mutations in genes with high penetrance (Mahdavi et al., 2019; Toland & Andreassen, 2017). It is a quite hard for a clinician to explain VUS information to patients and their families, once finding a VUS indicates that it was found "something" but it does not know what it means as far as the relationship to increased hereditary risk for developing certain cancers (Miller-Samuel et al., 2011). Because of this inability to classify *BRCA1/2* VUS as either benign or pathogenic and to determine which mutations are disease causing, which generates significant problems in risk evaluation, counselling and preventive care, several functional assays have been or are being developed (Carvalho et al., 2007). These type of assays work as independent classifiers of the VUS on their protein function and also provide additional data that in combination with available genetic and epidemiological data can predict cancer causality of VUS in a likelihood model (Carvalho et al., 2007).

Based on these assays and knowing that the process of cell division can be targeted to treat cancer patients, the use of genotoxic agents that initiate cell cycle checkpoints to target cancer cell proliferation is very useful, once they only cause DNA damage at high levels to treat cancer patients, which can lead to cell cycle arrest and/or cell death (Swift & Golsteyn, 2014). When these chemotherapeutics induce DNA lesions the cell must recognize and counter in order to survive using the DNA damage response (DDR), which will include DNA repair, suppression of global general translation, cell cycle arrest and, ultimately, either cell survival or cell death, as mentioned before (Woods & Turchi, 2013).

As described above, germline mutations in homologous recombination (HR) DNA repair genes (*BRCA1/2*) are linked to breast and ovarian cancer, once they play an important role in their function as regulators of DNA repair, transcription and cell cycle in response to DNA damage (Mahdavi et al., 2019). However, is not known that missense VUS are able to disrupt this important process. Because of that, VUS carriers diagnosed with metastatic breast or ovarian cancer might not benefit from therapy with platinum or PARP inhibitors targeting DNA-repair defects, as is currently recommended for individuals with *BRCA1* or *BRCA2* pathogenic variants (Guidugli et al., 2018; Tram, Savas, & Ozcelik, 2013). Thus, it is so important to use functional assays to analyse VUS, which allow to assess the cells response to repair the damage inflicted by genotoxic chemical agents.

1.8 CHEMOTERAPEUTIC AGENTS

Among the chemotherapeutics used, Doxorubicin, an anthracycline drug, is widely used to treat several cancers, including BC being one of many drugs that is contained in the respective chemotherapy cocktail regimen. It has multiple action mechanisms on cells: (i) intercalates and interferes with DNA

and RNA syntheses; (ii) inhibits topoisomerase II, which leads to strand breaks on DNA; and (iii) can cause the formation of ROS and free radical damage on cells. All of these effects inhibit cell proliferation, induce the G2-M cell arrest, or lead to apoptosis (Yurtcu, Işeri, & Sahin, 2014).

1.9 GOALS

The main aim of this study is based on the functional evaluation of the role of variants of unknown significance (VUS), previously identified in breast cancer patients and their relatives, in drug response. In order to achieve this goal, we used samples of peripheral blood lymphocytes from female volunteers to whom was identified the same VUS and belonging to high risk families. The study was carried-out using a genotoxic challenge through four techniques: TUNEL assay, Caspases activity assay, γH2Ax assay and Comet assay. With these methodologies we hypothesize that we might be able to characterize the VUS in cancer-related genes and give us some answers about its role in breast cancer risk.

2. MATERIALS AND METHODS

During all experimental procedures were used appropriate consumable material according the experimental steps and equipment's.

2.1 OVERVIEW

Over the years, genetic testing has been increasing for the identification of individuals and families with a genetic predisposition to developing breast cancer, the second most commonly diagnosed cancer. Consequently to this increase of tests, variants of uncertain significance (VUS) become more common in population. Once, these variants cannot be classified as either benign or pathogenic, the use of functional assays to assess the role of VUS may be helpful to identify its pathogenicity and characterization in cancer-related genes in breast cancer risk.

2.2 SAMPLE SELECTION

This study is composed by four female volunteers: two VUS-carriers for *BRCA1* gene (VUS-carrier 1 and VUS-carrier 2) and two negative controls (Control 1 and Control 2). The population enrolled was previously sequenced (Table 7.1), to confirm the variants, through NGS technology for a panel of BC specific genes described as essential on clinical guidelines for HBC studies, such as *BRCA1* and *BRCA2*.

Each volunteer agreed with the study and signed a consent document, represented in Figure 7.1 to Figure 7.3 of Appendix, for the use of samples for research, which it was approved by Ethical Commission of NMS/FCM (CEFCM). Furthermore, the VUS-carriers gave us detailed information about family history of oncological disease (Appendix - Figure 7.4).

2.3 PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

During the study the approaches were performed at least twice in independent experiments. For each single collection was drawn 20mL of peripheral blood by venous puncture. However, this amount is not enough to carried-out all experiments for once, which means that each donor had to draw blood for several times.

PBMCs were isolated through density gradient centrifugation using Histopaque-1077 (SIGMA, Darmstadt – Germany), a sterile ready-to-use medium that helps at recovery of viable lymphocytes and other mononuclear cells from the whole blood. Briefly, blood was diluted with an equal volume of phosphate-buffered saline (SIGMA, Darmstadt – Germany) (PBS) and then 5mL of this diluted blood were carefully added to a centrifuge tube, which already contain 3.5mL of Histopaque-1077, and centrifuged at 700g for 30min at room temperature. At the end of that time, PBMCs were harvested from the interface with a Pasteur pipette to a new centrifuge tube with 7mL of PBS, to wash the cells, and centrifuge again at 200g for 10min. After that, the pellet was suspended in RPMI 1640 medium (Gibco, Waltham, MA USA) supplemented with 25% of Fetal Serum Bovine (SIGMA, Darmstadt – Germany), or FBS, and 1.5% of Penicillin-Streptomycin (SIGMA, Darmstadt – Germany) (Pen-Strep) and then, the cells were ready to use.

2.4 BLOOD CELL CULTURE AND MUTAGEN TREATMENT

PBMCs were cultured in 12-well plates with RPMI 1640 medium supplemented with 25% of FBS and 1.5% of Pen-Strep. About 10^6 mononuclear cells were used in each assay, except γH2Ax assay that we used about 2×10^6 cells.

Mutagen treatment was performed with different incubation times, according to the optimization process of each assay. For each one was chosen three different concentration of Doxorubicin (Fisher BioReagents, Pittsburgh - USA), the genotoxic agent - 0.1, 1.0 and 5.0µM - and a positive (Camptothecin/H_2O_2) and a negative control (NC). Briefly, Camptothecin is an inhibitor of Type I topoisomerase that binds to DNA-TOP1 cleavage complexes, blocking re-ligation and resulting in the accumulation of SSBs, which leads to the activation of apoptosis, according to several studies (Swift & Golsteyn, 2014; Zeng et al., 2012), whereas reactive oxygen species (ROS), like H_2O_2, generated during oxidative stress only damage proteins, nucleic acids and cell membranes (Daroui, Desai, Li, Liu, & Liu, 2004). Because of that, Camptothecin was used to be a positive control on apoptosis assays (TUNEL and Caspases activity assays), and H_2O_2 a positive control on damage assays (comet assay).

2.5 FUNCTIONAL ASSAYS

TUNEL assay

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick- End Labeling (TUNEL) is an assay for detection of apoptotic DNA fragmentation *in situ*. TUNEL staining utilizes the ability of the enzyme terminal deoxynucleotidyl transferase (TdT) to incorporate labeled dUTP onto the free 3'-hydroxyl termini of fragmented genomic DNA, which can be visualized using immunohistochemical techniques (Kyrylkova, Kyryachenko, Leid, & Kioussi, 2012; Loo, 2011). Although TUNEL staining has been adopted as the method of choice for detecting apoptosis *in situ*, it is important to recognize that this assay is not limited to the detection of apoptotic cells, since this technique will label all free 3'-hydroxyl termini, regardless of the molecular mechanisms that led to the development of these termini, such as necrotic degenerating cells, cells undergoing DNA repair, cells damaged by mechanical forces and even cells undergoing active gene transcription. Thus, TUNEL staining should be considered generally as a method for the detection of DNA damage, and when used in combination with another apoptosis specific assay, more specifically as a method for identifying apoptotic cells (Loo, 2011).

Methodology

To carried-out TUNEL assay was used a commercial available kit by Thermo Fisher Scientific's APO-BrDU™ TUNEL Assay Kit. The methodology was performed according manufactures instructions, with minor alterations.

Briefly, after exposure to different concentrations of the genotoxic compound to be tested, for 4h at 37ºC with 5% CO_2, the samples were centrifuged at 200g for 5min and the respective supernatant was discarded. 500µL of PBS was added to each sample and centrifuged at 300g for 5min. After the removal of supernatant, the cells were resuspended in 500µL of Formaldehyde 2% (PBS; Formaldehyde 16% provided by Thermo Scientific, product of USA) and they were maintained for 15mins on ice. At the end of this time, the samples were centrifuged and each pellet was resuspended in 1mL of PBS and then, they are centrifuged again at 300g for 5min. The supernatant was removed, and the cells were resuspended in 500µl of PBS and 1mL of 70% cold Ethanol (in PBS) (PBS; Ethanol absolute provided by Merck, product of USA) and they were maintained for 30mins on ice. In the end, the samples were centrifuged to the same conditions and the supernatant removed. Then, the cells were resuspended and centrifuged twice with 1mL of Wash Buffer (provided in kit). Next, each pellet was resuspended in 50µL of DNA-labeling, which contains Reaction Buffer, TdT enzyme, BrdUTP and ddH_2O (each one, except ddH_2O, provided provided in kit), and they were kept over-night at 22ºC-24ºC.

By the next day, the samples were resuspended and centrifuged twice with 1mL of Rinse Buffer (provided in kit). After removed the supernatant the cells were counted to add 100µL of a diluted solution, which contains Thermo Fisher Scientific's anti-BrdU mouse monoclonal PRB-1 Alexa Fluor™ 488 conjugate (provided in kit) and Rinse Buffer, and then, they were incubated for 30min at room temperature, protected from light. Then, the samples were analysed by flow cytometry using a FACS Canto II Cytometer. Image analysis were performed using the FlowJo®.

Caspases activity assay

Caspases are a family of intracellular cysteine proteases and play distinct roles in apoptosis and inflammation (Kaufmann et al., 2008). Based on their functions, apoptotic caspases are classified as initiator caspases (caspases-2, -8, -9, and -10), which transduce various signals into proteolytic activity, and effector caspases (caspases-3, -6, and -7), which cleave most of the substrates that are degraded in cells and lead to cell disassembly and consequently to apoptosis (Kaufmann et al., 2008). A current model suggests that caspases 8, 9, and 10 can proteolytically activate procaspases 3 and 7, which are in turn responsible for proteolytically activating procaspase 6. Because various initiator caspases are activated by different signals, identifying the caspases that are activated and their order of activation after treatment of cells with a particular stimulus can provide insight into the lethal signalling that is induced by that stimulus (Kaufmann et al., 2008).

To detect active caspases, it is usually used Fluorochrome Inhibitors of Caspases (FLICA) that are cell permeable and non-cytotoxic. Once inside the cell, this inhibitor binds covalently to the active caspase, thereby inhibiting further enzymatic activity. Furthermore, once this inhibitor produces fluorescence, it is possible quantify the number of active caspases inside the cell by 96-well plate-based fluorometry, fluorescence microscopy, or flow cytometry.

Methodology

To carried-out Caspases assays were used a commercial available kit_by CHEMICON®'s CaspaTag™ Caspase-3/7 *In Situ* Assay Kit, and CaspaTag™ Caspase-9 *In Situ* Assay Kit. The methodology was performed according manufactures instructions, with minor alterations. The methodology used for both assays is the same the main difference is the FLICA concentration specific for each one.

Brief**y,** after exposure to different concentrations of the genotoxic to be tested, for 2h at 37ºC with 5% CO_2, the samples were centrifuged at 200g for 5min and after, the respective supernatant was discarded. The cells were resuspended in 200µL of PBS and it was added 10µL of FLICA 6× for Caspases 3/7 and FLICA 15× for Caspases 9 (PBS; FLICA 150×, provided in kit) to the respective tubes, and they were incubated 1h at 37°C with 5% CO_2, protected from light. During this time, the tubes were gently swirled 3 times, and after that, it was added 1mL of Wash Buffer 1× (Wash Buffer 10×, provided in kit) and gently mixed and then, they were centrifuged at 400g for 5min. Each pellet was resuspended in 1mL of Wash Buffer 1× and centrifuged again at 400g for 5min. After that time, the pellet was resuspended in 400µL of Wash Buffer 1× and it was added 2µL of Propidium Iodide (provided in kit), or PI, to the respective tubes, and after, the samples were analysed by flow cytometry using a FACS Canto II Cytometer. Image analysis were performed using the FlowJo®.

γH2Ax assay

The γH2Ax assay is a based analysis marker for DNA double-strand breaks (DSBs), which it is both relatively robust and practical to assess Chemical and IR-induced damage in cells (Kataoka, Bindokas, Duggan, Murley, & Grdina, 2006). Exposure to external factors results in the rapid phosphorylation of a minor nucleosomal histone protein, H2Ax. The phosphorylation will serve as a platform for the accumulation of many factors involved in the DNA damage response. The γH2Ax foci

formation is a sensitive and specific marker for the detection of DSBs that can be measure by fluorescent microscopy (Barnard et al., 2015; Dinis et al., 2012), but also by flow cytometry after a specific antibody reaction.

Methodology

After exposure to different concentrations of the genotoxic compounds to be tested, for 2h at 37ºC with 5% CO_2, the samples were centrifuged at 200g for 5min and the respective supernatant was discarded. The RPMI 1640 medium (1 ml) supplemented with 25% of FBS and 1.5% of Pen-Strep was added to each sample and incubated for 30min at 37ºC with 5% CO_2. After that time the samples were centrifuged and was added to the respective sample 1mL of PBS and 1µl of Violet Fluorescent Reactive Dye (provided by Thermo Fisher Scientific's LIVE / DEAD ™ Fixable Violet Dead Cell Stain Kit, product of USA) and they were incubated 30 minutes at room temperature, protected from light. Then, the samples were centrifuged to the same conditions and after discarding the supernatant, they were washed with 1 ml of PBS and centrifuged again. After that, the pellet was resuspended in 500µL of Formaldehyde 2% and the samples were maintained for 15min on ice. At the end of this time, they were centrifuged again and each pellet was resuspended in 500µL of 70% cold Ethanol (in PBS) and they were kept over-night at 4°C.

By the next day, the samples were centrifuged and each pellet was resuspended in 1 ml of Blocking Buffer, containing Albumin from Bovine Serum (BSA) 4% (SIGMA, Darmstadt – Germany) in PBS, 4% Goat Serum (SIGMA, product of USA) and 0.25% Triton X-100 (SIGMA, Darmstadt – Germany), and were further centrifuged. The supernatant was then removed and 5µL of antibody (Thermo Fisher Scientific's eBioscience ™ Anti-Hu/Mo pH2AX (S139) PE (product of USA)) were added to the respective samples, followed by 2h of incubation at room temperature, protected from light. After that, the cells were washed with 1.5mL of BSA 1% and centrifuged. Then, each pellet was resuspended in 200µl of BSA 0.1% and after, the samples were analysed by flow cytometry using a FACS Canto II Cytometer. Image analysis were performed using the FlowJo®.

Comet assay

The Comet Assay, also called single cell gel electrophoresis (SCGE), is a sensitive and rapid technique for quantifying and analysing DNA damage in individual cells. The term *comet*, resulted from their appearance, is used to identify the single cell DNA migration. Briefly, the cells are embedded in agarose and then they are placed on a microscope slide. Following the slide preparation, the embedded cells are lysed by detergents and high salt, which promote the removal of all cellular proteins from the cells. Then, they are electrophoresed under alkaline conditions, allowing the broken DNA fragments or damaged DNA to migrate away from the nucleus, after which it is quantified by staining. The extent of free DNA from the *head* to *tail* of the comet is directly proportional to the amount of DNA damage (Fairbairn, Olive, & O'Neill, 1995; Tice et al., 2000).

Comparing with another genotoxic assays, the comet assay: demonstrate sensitivity for detecting low levels of DNA damage, require a small number of cells per sample, is flexible and low cost, use small amounts of test substance and it's only needed a short period of time to complete an experiment (Tice et al., 2000).

Methodology

After exposure to different concentrations of the genotoxic compound under test, for 2h at 37ºC with 5% CO_2, the samples were centrifuged at 200g for 5min and the respective supernatant was discarded. PBS (1 ml) was added to each sample and centrifuged again. The pellet was resuspended in 100µL of 0.5% Agarose Low Melting Point (PBS; Agarose provided by Bioline, London UK) and 40µL of each suspension were spread on microscope slides that were previously coated with 1% Agarose

Normal Melting Point. Next, they were kept for 20min at 4ºC and after that, the slides were left overnight in cold-lysis buffer (2.5M NaCl (Fisher Scientific, Leicestershire); 100mM EDTA(SIGMA, Darmstadt – Germany); 10mM Tris Base (Fisher Scientific, Leicestershire); 1% Triton X-100; pH 10), protected from light.

By the next day, the cold-lysis buffer was removed, and the slides were washed with cold-double-distilled water and kept in it for 10min at 4ºC, protected from light. After that, they were immersed in cold-electrophoresis buffer (200mM EDTA; 10M NaOH (Acros Organics, Geel - Belgium.); pH≥13) for 20min at 4ºC, protected from light. After that time, the electrophoresis occurred at 25V for 20min and then, the slides were neutralized three times for 5min each with a neutralization buffer (0.4M Tris (SIGMA, Darmstadt – Germany); pH 7.5). Next, they were dried with Ethanol 50%, 75% and 100%, 5min each, and left in a hot grid to completely dry. After, they were stained with GelRed Nucleic Acid Stain 3× (GelRed Nucleic Acid Stain 10.000× provided by Biotium, Fremont, CA) and observed with a Fluorescent Microscope (Zeiss Z2) using an ampliation of 200x, where we randomly selected 50 images from each slide, giving a total of 200 cells analyzed per data point. The percentage of DNA in Tail (% DNA in Tail) of comets was measured to assess the extent of DNA damage. Image analysis were performed using the CometScore™.

2.6 BIOINFORMATICS AND STATISTICAL ANALYSIS

The statistical analyses were carried out using GraphPad Prism 5 (GraphPad Software). Data were plotted as mean expression ± the standard deviation (SD) of replicates. The two-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's multiple comparisons) were performed for TUNEL assay, Caspases activity assay and γH2Ax assay, to compare between doses of Doxorubicin, positive controls and non-treated cells (0μM) and to compare between the different groups of samples. The one-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's multiple comparisons) were performed for Comet assay to compare between doses, positive controls and non-treated cells (0μM), and to compare between the different groups of samples it was used the one-way analysis of variance (ANOVA) and post-hoc testing (Tukey's pairwise comparisons).

3. RESULTS

3.1 TUNEL assay

To analyse the apoptosis of the cells and to clarify if VUS carrier cells had a different response to damage induced by genotoxic agents, we firstly determined the concentration range of Doxorubicin to damage or cause death to PBMCs. Through that, we used three different concentrations (0.1µM, 1µM and 5µM) and analysed the samples by flow cytometry. For each sample, we isolated the population of interest and applied the flow cytometer's parameters that correspond with the antibody fluorescence that we used for TUNEL assay (Appendix - Figure 7.5 A). These results are represented in Figure 3.1 and Figure 3.2, where is possibly to observe an increase percentage of cells that suffered apoptosis (TUNEL-positive cells) in all samples with increasing concentration of drug. This distribution of TUNEL-positive cells is represented by mean+SD and it was used the 2way ANOVA and Bonferroni post-tests to compare the percentage of positive cells between samples (Figure 3.1) and between doses in each sample (Figure 3.2). While between samples no difference statistically significant was observed for all samples, in between doses, VUS-carrier 2 and Control 1 and 2 showed statistical significance (p-value<0.05) when comparing 5µM Dox with the negative control (NC - 0µM), and only Control 1 showed statistical significance (p-value<0.05) when comparing 10µM CPT with the negative control (NC - 0µM).

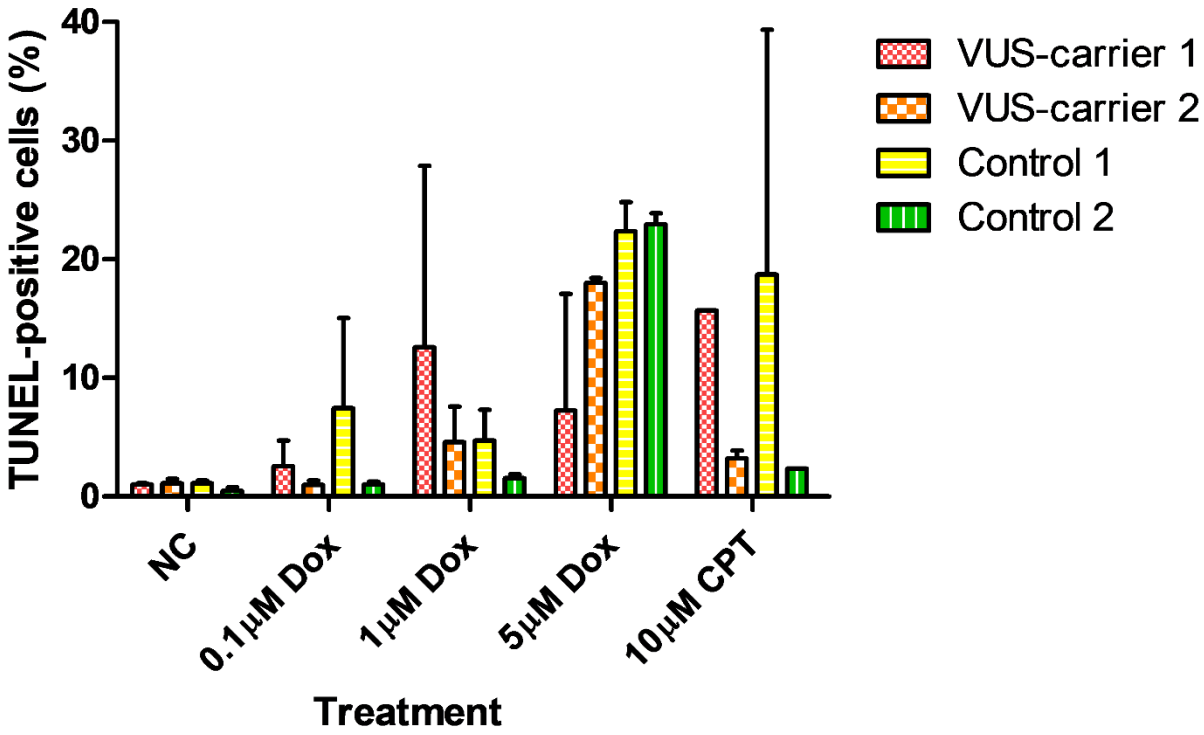

Figure 3.1 - Frequency of TUNEL-positive cells (%) for each dose of Doxorubicin. Results are expressed as mean+SD. Statistical analysis was performed with GraphPad Prism 5, using the two-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's multiple comparisons).

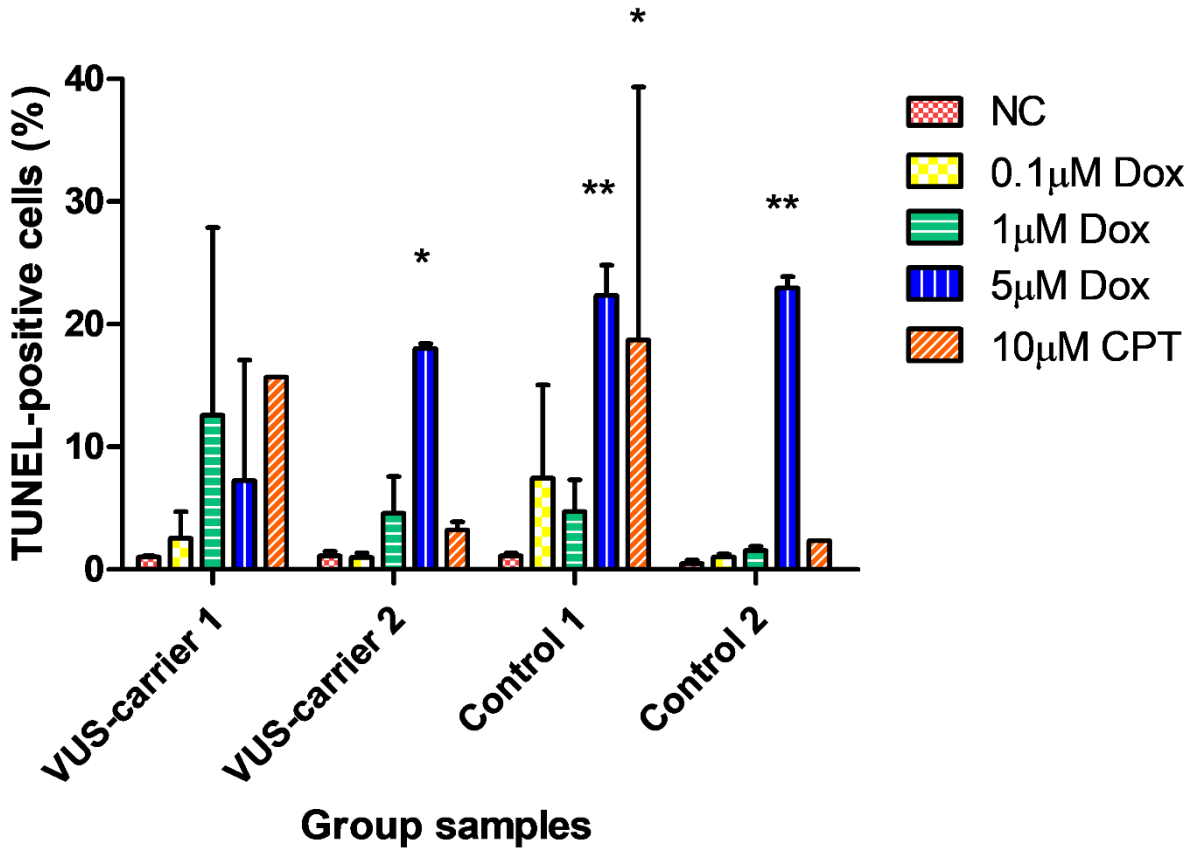

Figure 3.2 - Frequency of TUNEL-positive cells (%) for each sample. Results are expressed as mean+SD. Statistical analysis was performed with GraphPad Prism 5, using the two-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's multiple comparisons). *p<0.05; **p<0.01, ***p<0.001 in relationship to the negative control (NC - 0µM).

3.2 Caspases activity assay

Like TUNEL assay, this technique allowed us to analyse the apoptosis of PBMCs through three different concentrations of Doxorubicin and its samples were analysed by flow cytometry. After we got the isolated population and the correct flow cytometer's parameters (Appendix - Figure 7.5 B), we represented its results in Figure 3.3 to Figure 3.8. First, we analysed the caspases-3/7 activity (Figure 3.3 and Figure 3.4) and then, the caspases-9 activity (Figure 3.5 and Figure 3.6).

In these two assays, we analysed the apoptotic rate (early and late apoptosis) *vs* necrotic rate and they were represented by mean+SD and it was used the 2way ANOVA and Bonferroni post-tests to compare the samples, represented by Figure 3.3 and Figure 3.5, and between doses in each sample, represented by Figure 3.4 and Figure 3.6. These results showed us statistical significance (p-value<0.05) in 0.1µM of Doxorubicin between samples (Figure 3.3) and in 1 and 5µM of Doxorubicin between doses on VUS-carriers 1 and 2 in caspases-3/7 activity (Figure 3.4). In caspases-9 activity there was only statistical significance (p-value<0.05) in VUS-carrier 2 when comparing the concentrations of 1 and 5µM of Doxorubicin with the negative control (NC - 0µM), represented by Figure 3.6.

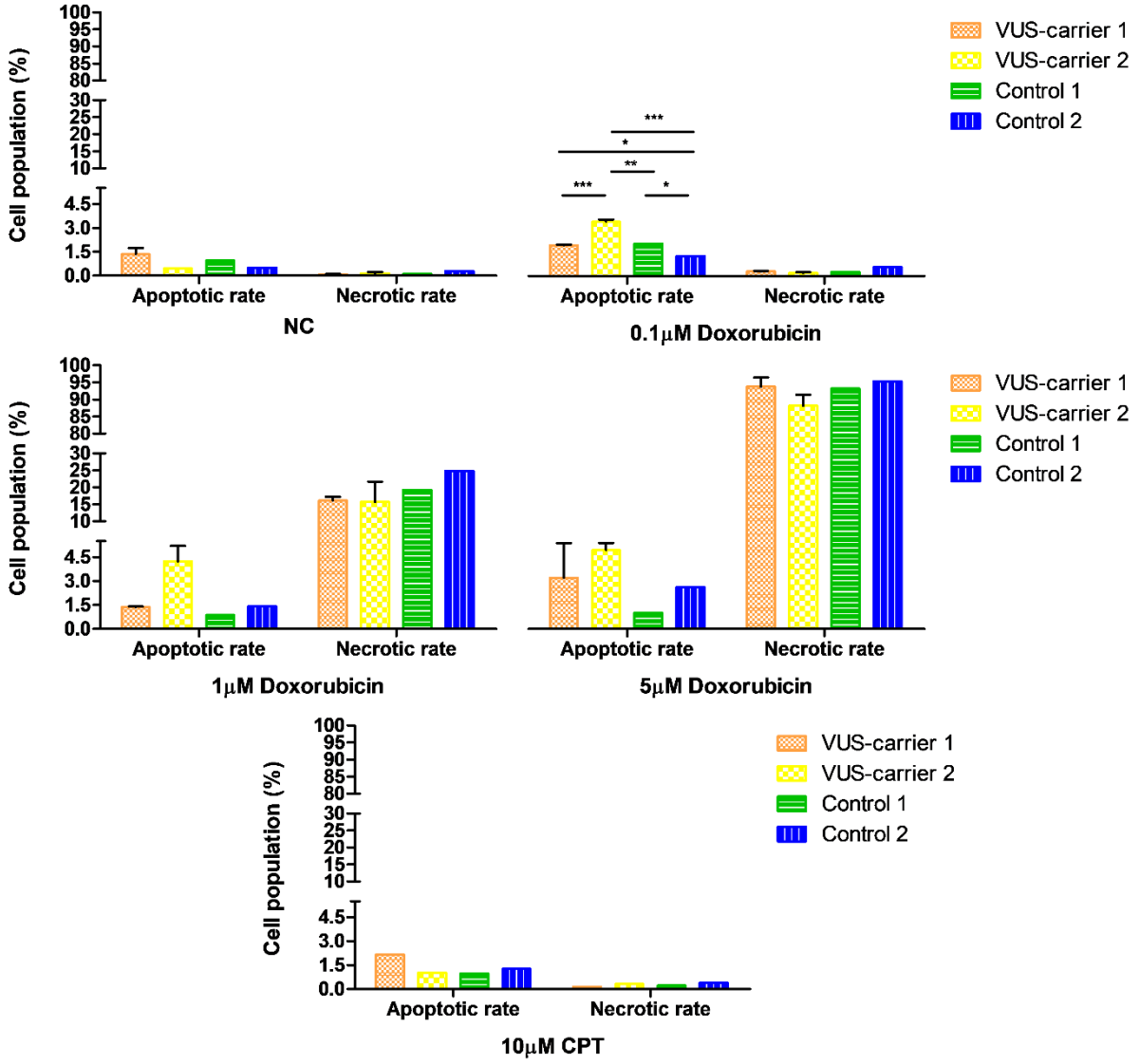

Figure 3.3 - Frequency of cell population (%) for each dose of Doxorubicin with Apoptotic status and Necrotic status, in Caspases-3/7 assay. Results are expressed as mean+SD. Apoptotic rate includes Early and Late Apoptosis and Necrotic rate only includes Necrosis. Statistical analysis was performed with GraphPad Prism 5, using the two-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's multiple comparisons). *p<0.05; **p<0.01, ***p<0.001, in relationship to another sample.

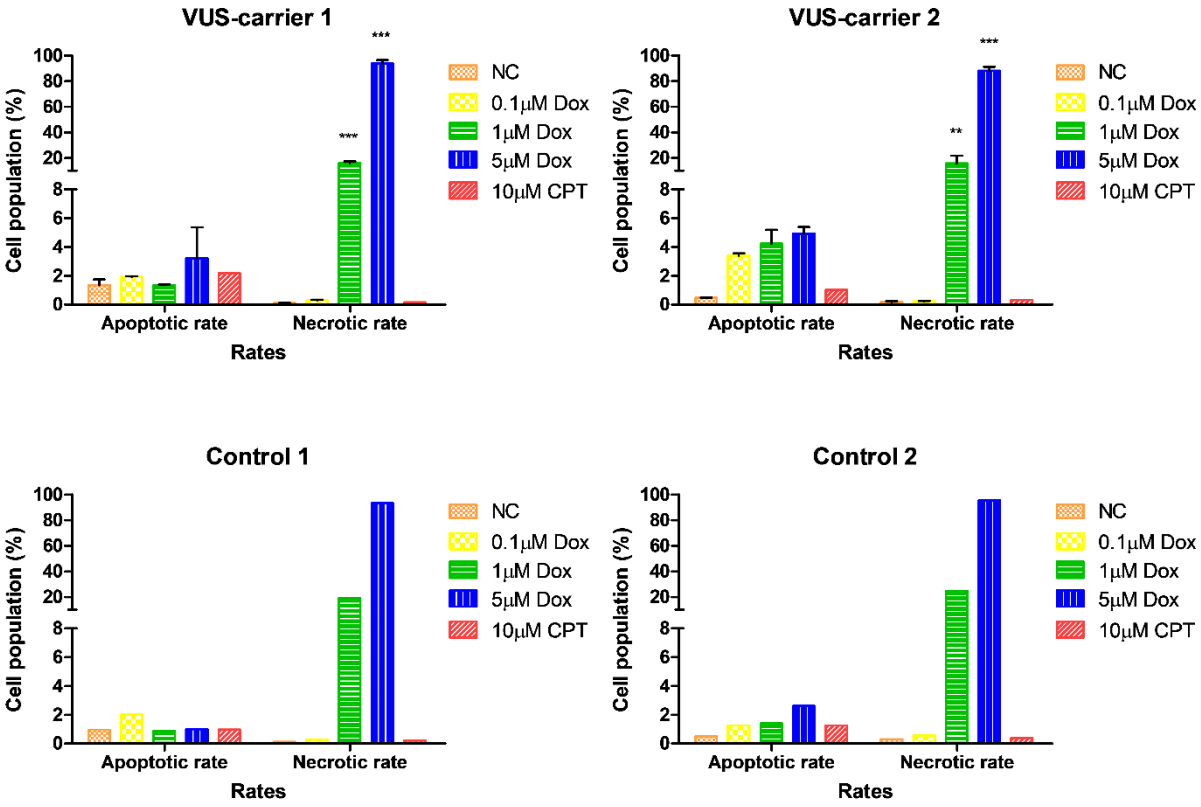

Figure 3.4 - Frequency of cell population (%) for each sample with Apoptotic status and Necrotic status, in Caspases-3/7 assay. Results are expressed as mean+SD. Apoptotic rate includes Early and Late Apoptosis and Necrotic rate only includes Necrosis. Statistical analysis was performed with GraphPad Prism 5, using the two-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's multiple comparisons). **p<0.01, ***p<0.001, in relationship to the negative control (NC - 0μM).

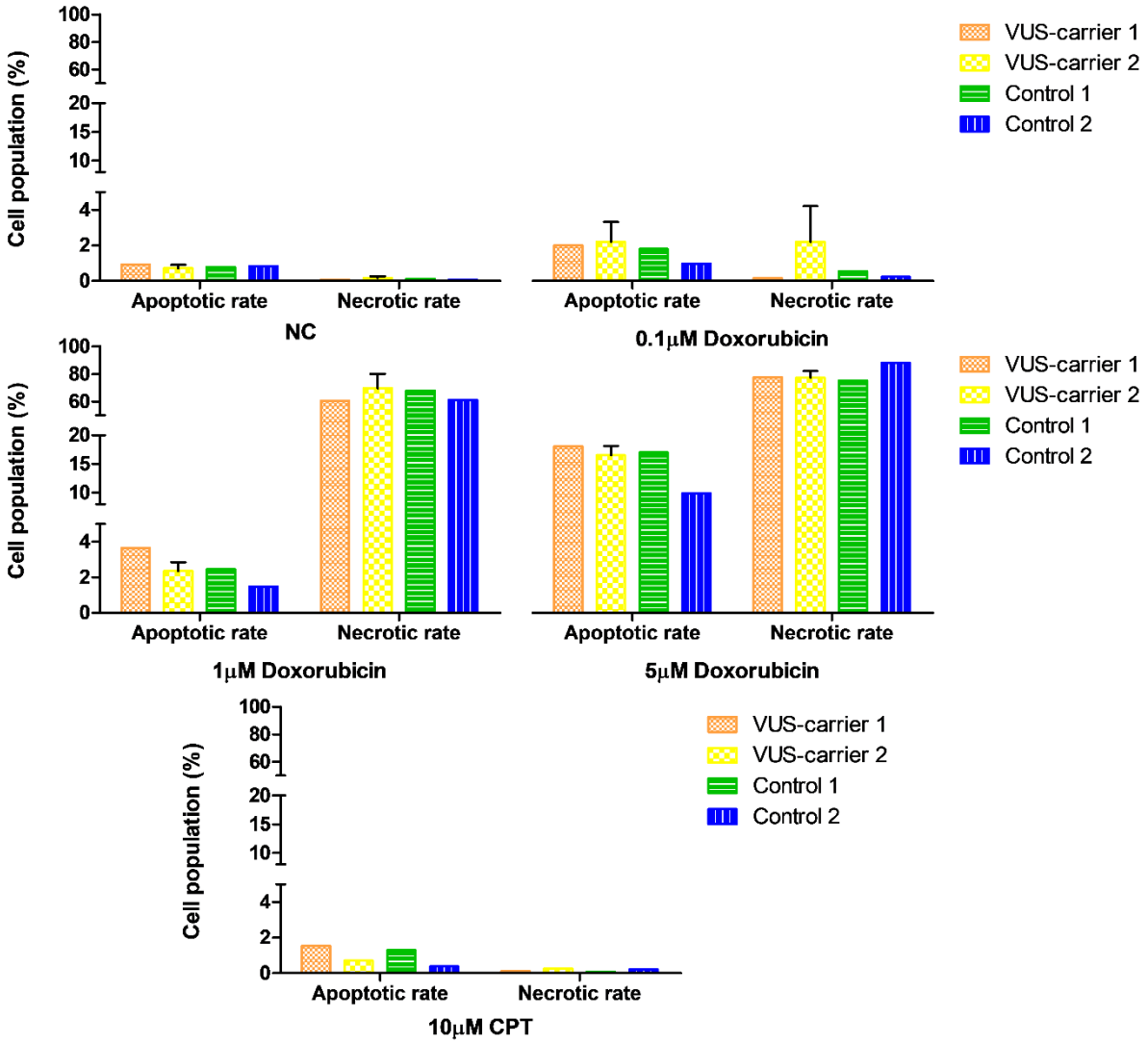

Figure 3.5 - Frequency of cell population (%) for each dose of Doxorubicin with Apoptotic status and Necrotic status, in Caspase-9 assay. Results are expressed as mean+SD. Apoptotic rate includes Early and Late Apoptosis and Necrotic rate only includes Necrosis. Statistical analysis was performed with GraphPad Prism 5, using the two-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's multiple comparisons).

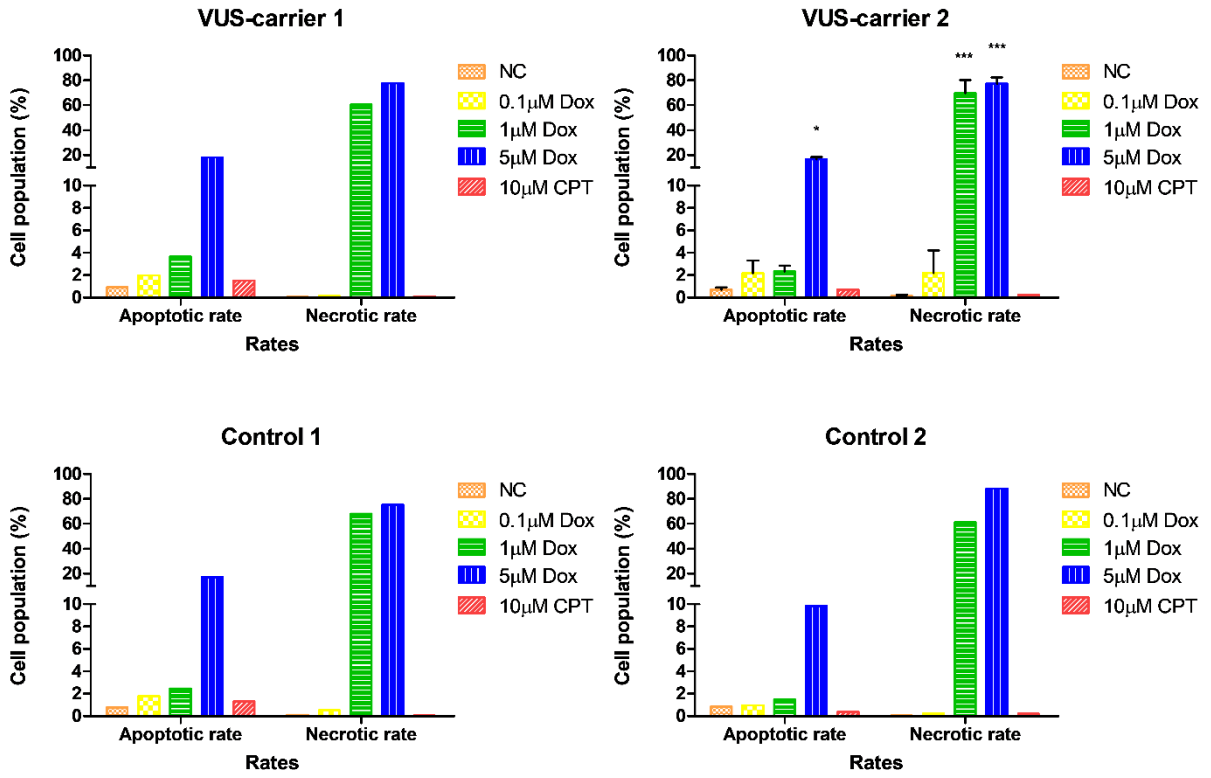

Figure 3.6 - Frequency of cell population (%) for each sample with Apoptotic status and Necrotic status, in Caspase-9 assay. Results are expressed as mean+SD. Apoptotic rate includes Early and Late Apoptosis and Necrotic rate only includes Necrosis. Statistical analysis was performed with GraphPad Prism 5, using the two-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's multiple comparisons). *p<0.05, ***p<0.001, in relationship to the negative control (NC - 0µM).

Therefore, we linked these two assays to compare between them, using the percentage of apoptosis. As showed in Figure 3.7 there was difference statistically significance on VUS-carrier 1, VUS-carrier 2 and Control 1 with 5µM of Doxorubicin (p-value<0.05), but all of them showed an increase of apoptosis with increasing the concentration of the drug. Furthermore, in Figure 3.8 is observed statistical significance (p-value<0.05) at caspase-9 and both caspases-3/7 and caspase-9, respectively in VUS-carrier 1 and VUS-carrier 2, for 5µM of Doxorubicin when comparing with NC (0µM).

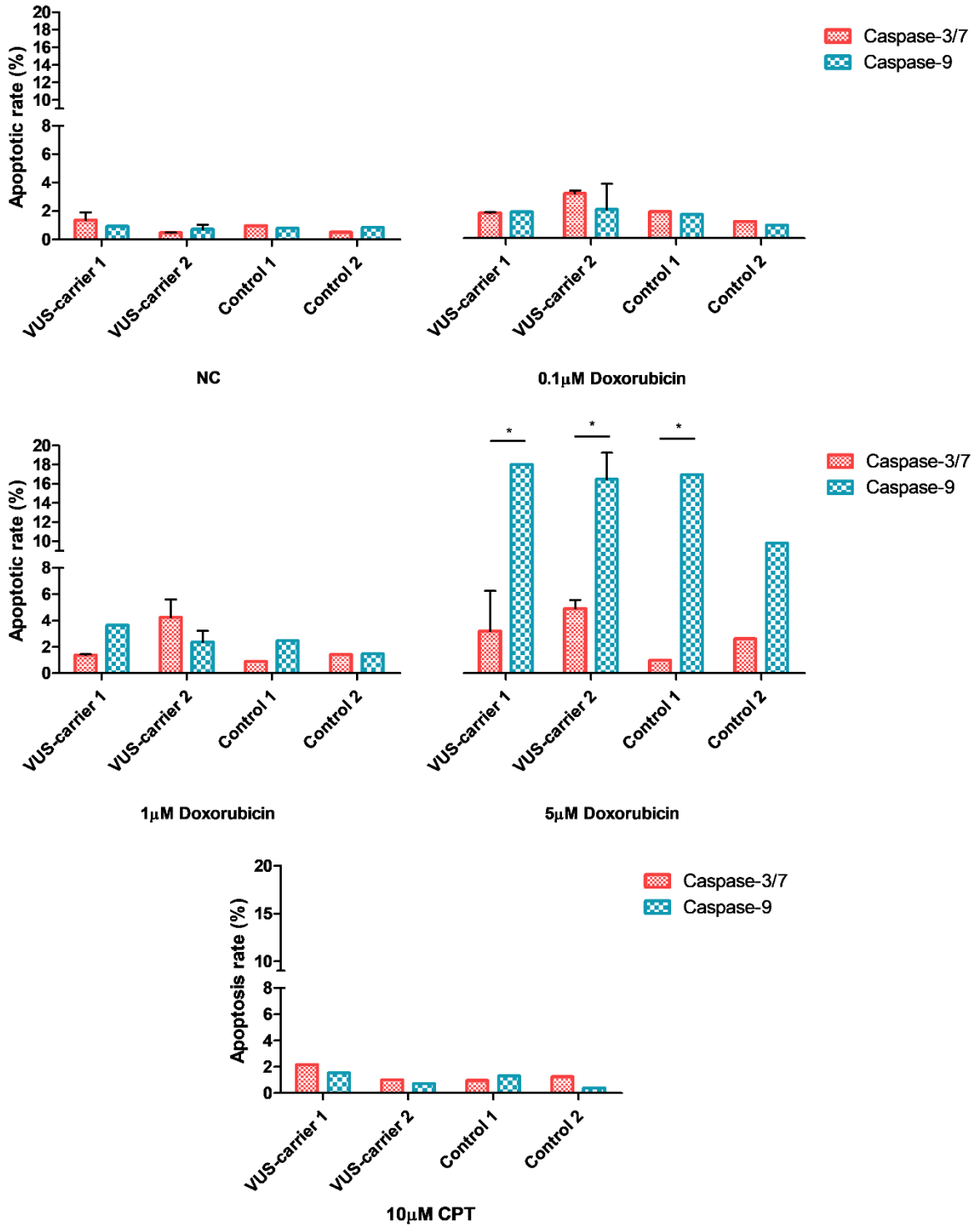

Figure 3.7 - Frequency of apoptotic cells (%) for each dose of Doxorubicin in Caspases-3/7 and 9 assays. Results are expressed as mean+SD. Statistical analysis was performed with GraphPad Prism 5, using the two-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's multiple comparisons). *$p<0.05$, in relationship to another sample.

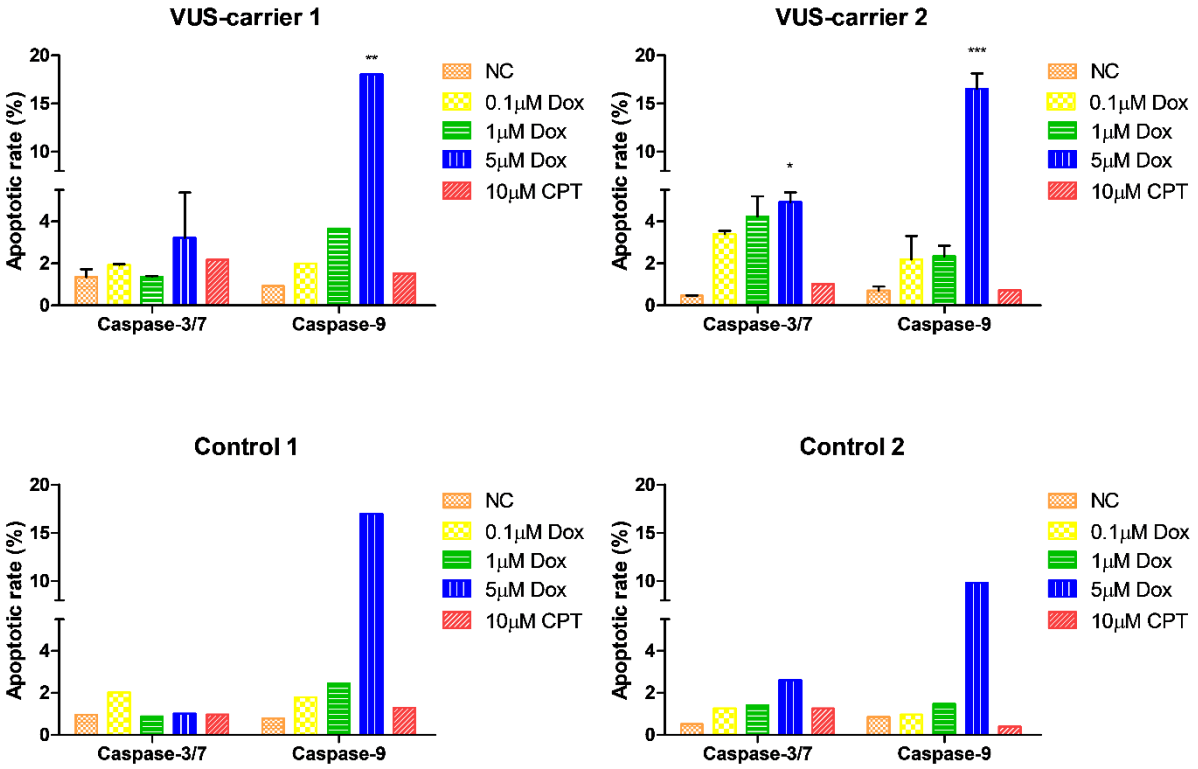

Figure 3.8 - Frequency of apoptotic cells (%) for each sample in Caspases-3/7 and 9 assays. Results are expressed as mean+SD. Statistical analysis was performed with GraphPad Prism 5, using the two-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's multiple comparisons). *p<0.05; **p<0.01, ***p<0.001, in relationship to the negative control (NC - 0µM).

3.3 γH2Ax assay

This technique allowed us to analyse the damage and its respective response to different concentrations of Doxorubicin, like the other assays, in PBMCs from two VUS-carriers and two controls, and the respective samples were analysed by flow cytometry. After we got the isolated population and the correct flow cytometer's parameters (Appendix - Figure 7.6), we represented its results in Figure 3.9 and Figure 3.10, where it is possible to observe an increase of cells that suffered serious damage, which caused the phosphorylation of γH2Ax histone, in all samples when the drug is used, and according with the rise of its concentration. This distribution of γH2Ax positive cells is represented by mean+SD and it was used the 2way ANOVA and Bonferroni post-tests to compare the percentage of positive cells between samples (Figure 3.9) and between doses in each sample (Figure 3.10). While in between samples no difference statistically significance was observed, except in comparison of VUS-carrier 2 with Control 2 (p-value<0.05), in between doses each sample showed statistically significance (p-value<0.05) when comparing 5µM with NC (0µM).

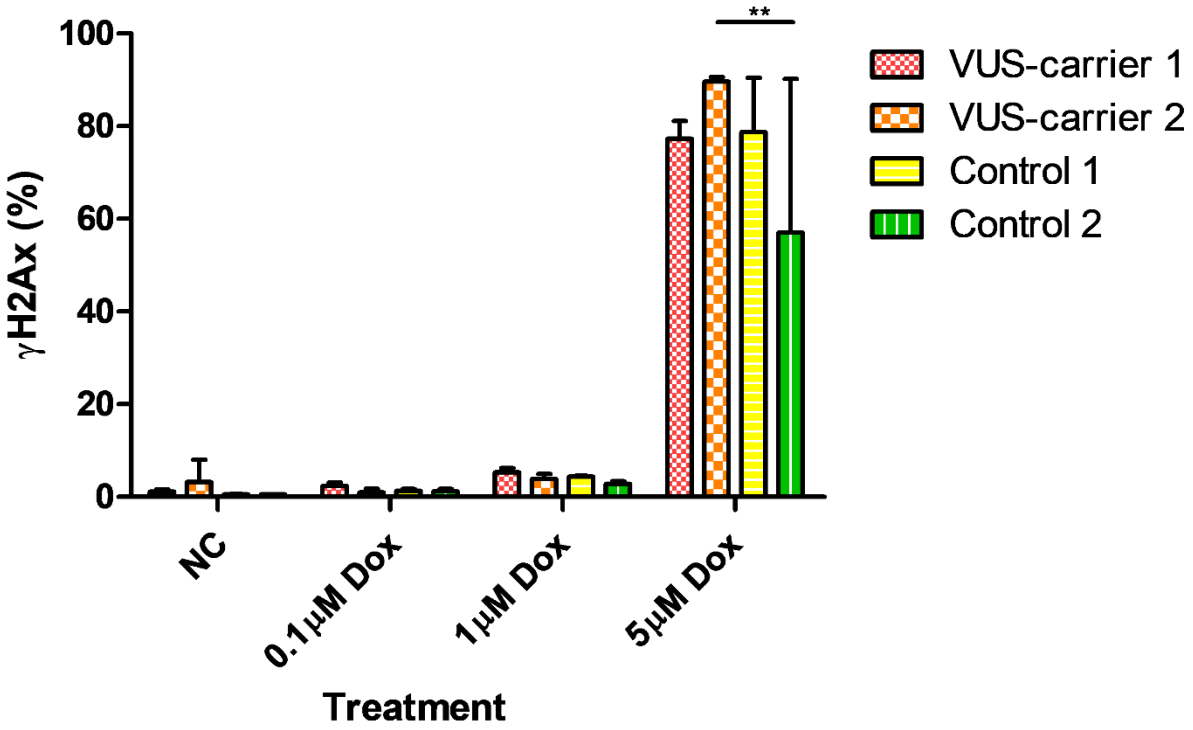

Figure 3.9 - Frequency of γH2Ax (%) for each dose of Doxorubicin. Results are expressed as mean ± SD. Statistical analysis was performed with GraphPad Prism 5, using the two-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's multiple comparisons). **p<0.01 in relationship to another sample.

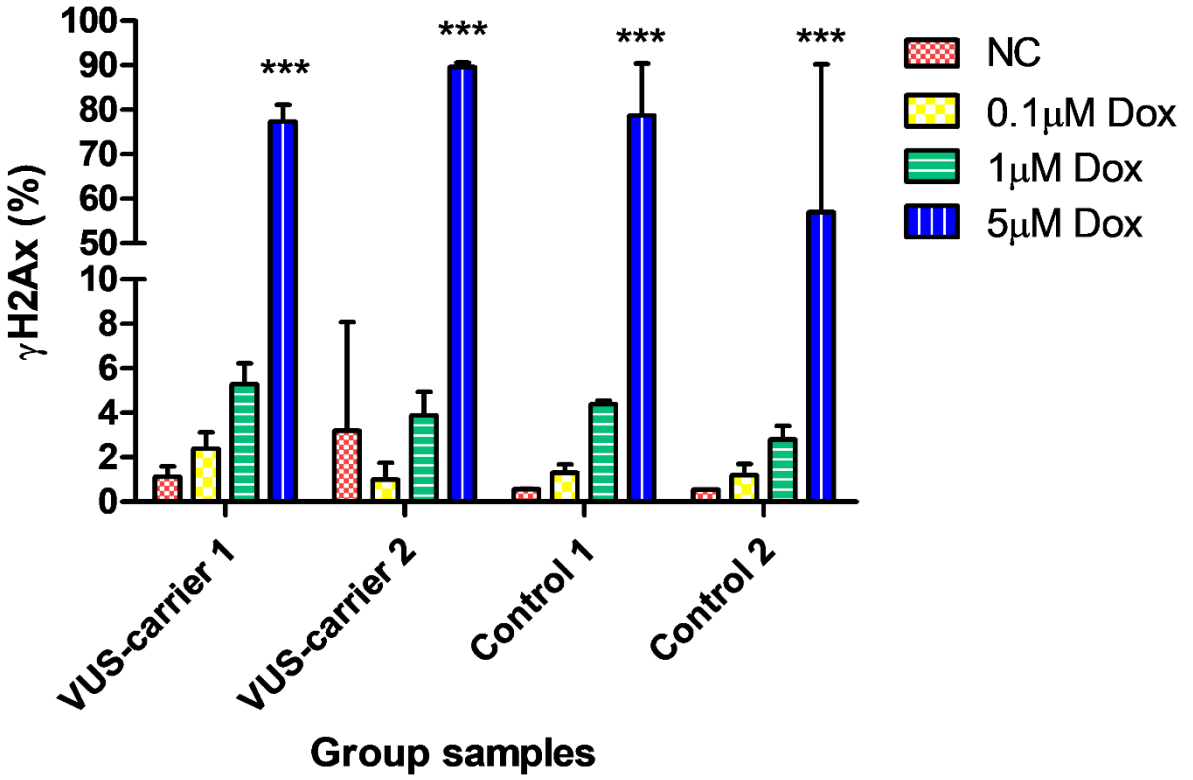

Figure 3.10 - Frequency of γH2Ax (%) for each sample. Results are expressed as mean+SD. Statistical analysis was performed with GraphPad Prism 5, using the two-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's multiple comparisons). ***p<0.001 in relationship to the negative control (NC - 0μM).

3.4 Comet assay

Like γH2Ax assay, this technique allowed to analyse the response to damage in PBMCs through different concentrations of Doxorubicin and the samples were analysed by fluorescent microscopy with 200x ampliation. In each slide, we randomly select 50 images, giving a total of 200 cells on each dose, represented on Figure 3.11. In some cases, the number of cells was not reached, but the maximum limit was kept on 200 cells. After we got the images, each one of them was analysed by the CometScore Software that returned the percentage of DNA in Tail (% DNA in Tail) of the cells. This distribution of DNA in Tail is represented by mean and it was used the 1way ANOVA and, Tukey and Bonferroni post-tests to compare the percentage of DNA in Tail between samples (Figure 3.12) and between doses in each sample (Figure 3.13). In Figure 3.12 it was observed statistical significance (p-value<0.05) between some samples in each dose, except 1µM of Doxorubicin. Furthermore, in Figure 3.13 it was observed difference statistically significance (p-value<0.05) between some doses in all samples, except in VUS-carrier 2. It is important to mention that this was the only assay, where we performed one independent experiment for each sample.

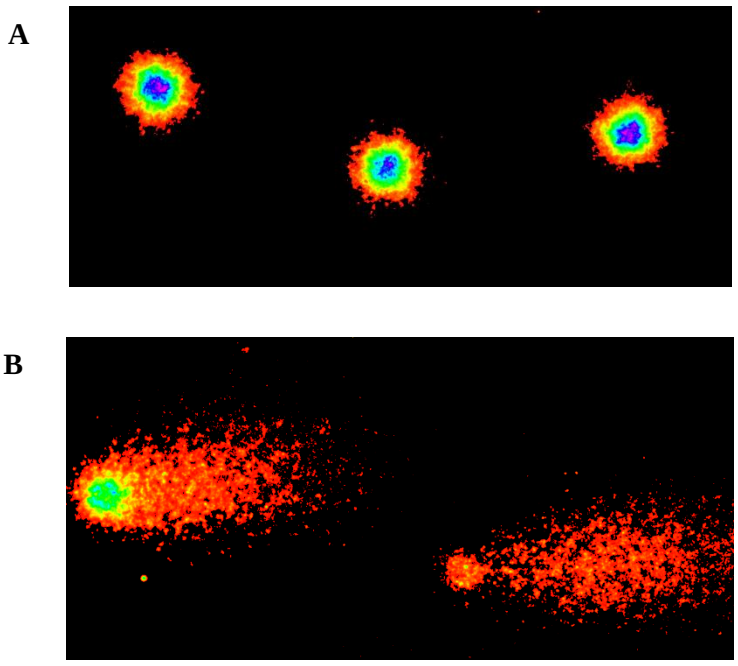

Figure 3.11 - Representative images of cells captured for comet assay. (A) Normal cells with DNA in nucleus; (B) Damaged cells with migration of DNA creating "comets".

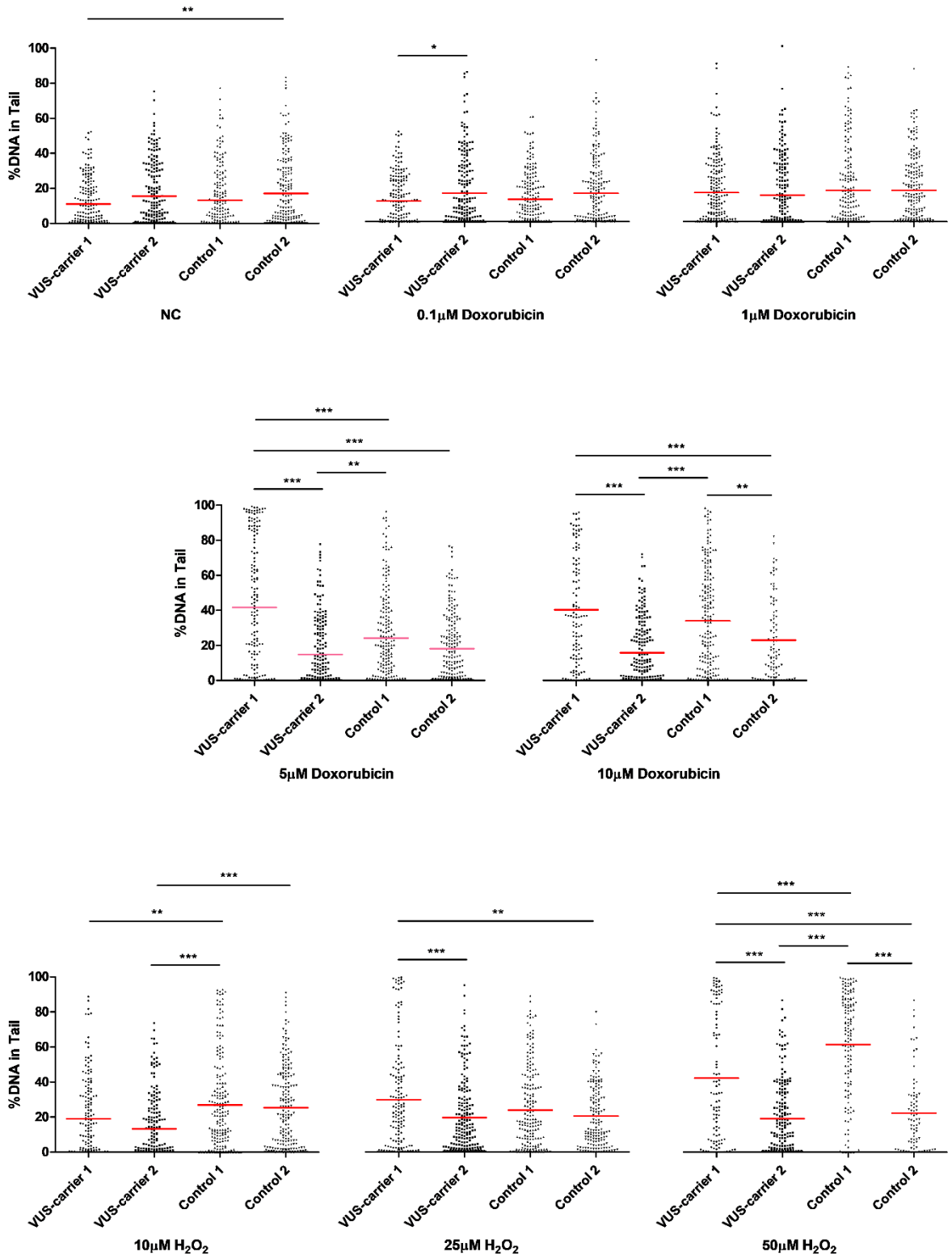

Figure 3.12 - Distribution of DNA in Tail (%) for each dose of Doxorubicin. Results are expressed as mean ± SD. Statistical analysis was performed with GraphPad Prism 5, using the one-way analysis of variance (ANOVA) and post-hoc testing (Tukey's pairwise comparisons). *p<0.05; **p<0.01, ***p<0.001 in relationship to another sample.

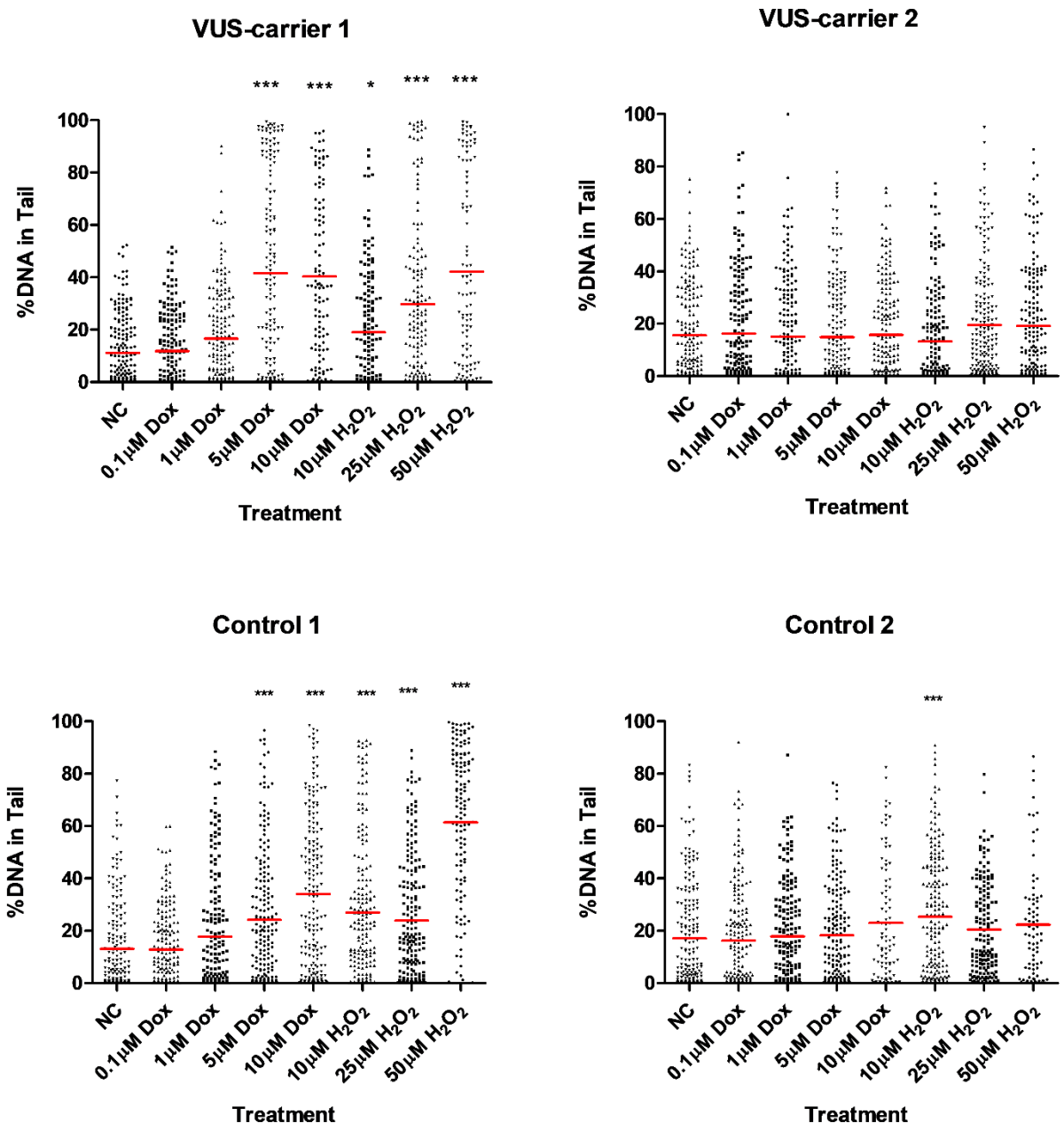

Figure 3.13 - Distribution of DNA in Tail (%) for each sample. Results are expressed as mean+SD. Statistical analysis was performed with GraphPad Prism 5, using the one-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's multiple comparisons). *p < 0.05; ***p<0.001 in relationship to the negative control (NC - 0µM).

4. DISCUSSION

Nucleic acids are the most sensitive biologic molecules to chemical reactions, which means that when seriously damage might contribute to increase the genomic instability in cells (Hoeijmakers, 2009). Particularly in DNA, this damage can be from endogenous and exogenous agents that consequently involve one (SSB) or both (DSB) strands of the molecule. To avoid genomic instability, one of the hallmarks of cancer, DNA damage is repaired by DDR or damaged cells are eliminated by death mechanisms (Nowsheen & Yang, 2013; Roos & Kaina, 2006). The most important error-free DNA DSB repair mechanism is HR that align DSBs ends prior to ligation and an alteration in this pathway, mainly promoted by *BRCA1* mutation, it destabilizes the genomic maintenance and confer an increased risk of developing breast cancer (Helena et al., 2018; Mahdavi et al., 2019; Roos & Kaina, 2006). To overcome the disease is essentially to perform genetic tests that identify individuals and families with higher genetic predisposition to develop cancer, such as *BRCA1* and *BRCA2*. One of the possible results from genetic tests is VUS, or variants of uncertain significance, that cannot be classify as benign or pathogenic alteration, which generates significant problems in risk evaluation, counselling and preventive care (Carvalho et al., 2007; Hoffman-Andrews, 2017; Mahdavi et al., 2019). With this in mind, we performed four functional assays that enable to assess cell response to repair the damage inflicted by genotoxic agents in peripheral blood lymphocytes from two VUS-carriers patients in the BRCA1 gene (VUS-carrier 1 and 2) and two controls (Control 1 and 2), without any variant detected after sequencing analysis. These samples were treated with Doxorubicin, an anthracycline drug widely used to treat several cancers, which intercalates and interferes with DNA and RNA syntheses, inhibits topoisomerase II, which leads to strand breaks on DNA, and can cause the formation of ROS and free radical damage on cells, and all of them, furthermore, lead to apoptosis (Yurtcu et al., 2014). Then the response to damage was assessed by TUNEL assay, Caspases activity assay, γH2Ax assay and Comet assay.

TUNEL assay is a method used for localization of apoptotic DNA fragmentation *in situ*, although it can be used as well as an assay for the detection of DNA damage (Kyrylkova et al., 2012; Loo, 2011). With this technique it was possible to observe, in Figure 3.2, statistical significance for 5µM of Doxorubicin at VUS-carrier 2 and both Controls 1 and 2, when comparing with negative control (NC - 0µM), and an increase of TUNEL-positive cells from negative control (NC - 0µM) to 5µM of Doxorubicin only in VUS-carrier 2 and Control 2 and not in all of them as expected, which can be explained by intra-individual variability that exist in this study, proved by the high SD observed in VUS-carrier 1 and Control 1. Furthermore, we worked with living cells (PBMCs) from different persons in different time periods, which exponentially increase this variability, along with other aspects of the human conditions that can influence the normal gene expression (Whitney et al., 2003). When observing the graph in Figure 3.1, we do not observe any difference statistically significance, but we can observed that both Controls 1 and 2 had the highest population of positive cells, compared with VUS-carriers 1 and 2. This is also observed in micronuclei assay described in the master's thesis "Functional Characterization of Variants of Unknown Significance in Familial Breast Cancer" by Rita Lourenço, a previous work developed by our group. Based on that, due to the acute exposure of genotoxicity employed in blood cells, they do not have the skill to repair the damage inflicted and thus, there is an excessive amount of genome damage that accumulated. And so, cells with that excessive damage are eliminated by apoptosis (Bonassi et al., 2007). Due these results it is possible to assume that cells from VUS-carrier 1 and 2, with an excessive genome damage are eliminated by apoptosis, but less than the quantity of apoptotic cells provided by Control 1 and 2. If a VUS from *BRCA1* gene had biologic meaning these results are not the expected, once Control 1 and 2 had, theoretically, less sensitivity to damage than VUS-carrier 1 and 2, guiding to a lower apoptosis activity, but once we do not know its

biologic meaning, this assay should be perform once again, and with more apoptosis assays to see what is it will be the correct answer. Additionally, our positive control of drug (10µM CPT), only show statistical difference, in Figure 3.2, for Control 1 and with a higher SD. This is not expected, once 10µM CPT should be able to promote the apoptosis of cells at highs levels, and not minimum levels as we see. To eliminate the variability that can mask the results and to ensure the veracity of the results, more assays should be performed in the future, and maybe with a dose of CPT more efficient.

The caspases activity assay has been used to detect active caspases, proteases linked to apoptosis, which allow to quantify cells that suffered serious damage leading to a higher level of apoptotic cells (Kaufmann et al., 2008). When analysing the activity of capases-3/7 in Figure 3.4, it is possible to see an increase of apoptotic activity by increasing the concentration of Doxorubicin for all samples, except Control 1, not showing any difference statistically significance between the doses. However, when comparing between samples, in Figure 3.3, they show statistical significance (p-value<0.05) in 0.1µM of Doxorubicin between all samples, except between VUS-carrier 1 and Control 1. This result may indicate a sensitivity of samples at lower doses of Doxorubicin, and once again the existence of genetic variability, in this case inter-individual variability. Additionally, the positive control for apoptosis (10µM CPT) does not show high levels of apoptosis compared with Doxorubicin. However, it is observed that the levels of apoptosis is higher than the necrotic levels, what is expected, once CPT promote cellular apoptosis, as mentioned before (Swift & Golsteyn, 2014; Zeng et al., 2012).

In caspase-9 activity analysis, when observing Figure 3.6, there is only statistically significance (p-value<0.05) in apoptotic activity at higher doses of Doxorubicin in VUS-carrier 2, however it is visible that apoptosis increases by increasing the concentration of Doxorubicin for all samples. Furthermore, in Figure 3.5 it is possible to observe that there is not statistical significance between samples, which may indicate that both VUS-carriers and Controls do not have a higher difference at apoptosis activity. However, Controls have less apoptosis than the VUS-carriers, and because of that it is necessary to perform more assays to confirm our observational data. Additionally, the positive control for apoptosis (10µM CPT) does not show high levels of apoptosis compared with Doxorubicin, like the Caspases-3/7 assay, but show as the last one, high levels of apoptosis compared with necrotic rate.

When combining these two caspases activity assays, it is possible to see, in Figure 3.7, that both caspases-3/7 and caspase-9 do not have statistical difference until 5µM of Doxorubicin, where caspase-9 have a higher apoptotic activity when comparing with caspases-3/7, with a statistically significance (p-value<0.05) in all samples, except Control 2. This is explained by caspases pathway, where pro-caspase-9 is recruited and activated by the apoptosome to active effector caspases, like caspase-3 and -7, and trigger a cascade of events leading to apoptosis. So, it is this bonding between pro-caspase-9 and apoptosome that promote the significant activity observed in this figure. Additionally, once caspases-3/7 are later activated and they not remain binding to apoptosome, its activity decrease (Li et al., 2017; Wu & Bratton, 2017).

Additionally, when analysing the influence of Doxorubicin in Figure 3.8, VUS-carriers 1 and 2 showed a statistical difference (p-value<0.05) with negative control (NC - 0µM) at 5µM of Doxorubicin in both caspases, except caspases-3/7 in VUS-carrier 1. Neither of the Controls shows statistical difference after the treatment, which it is expected and might be associated with less sensitivity to the drug than that exists in BRCA1 VUS cells, where maybe there is a defective in DNA repair by homologous recombination. Furthermore, 10µM CPT does not show high levels of apoptosis, compared with Doxorubicin, and being the positive control for apoptosis it is not expected these results. Therefore, in future assays it is essential to use a dose more efficient of CPT, or even another drug to be our positive control.

The apoptotic activity obtained by TUNEL assay and by Caspases activity assay shows a difference between the results. While, at higher concentrations in TUNEL assay there is a bigger value of apoptosis for Controls 1 and 2, compared with VUS-carriers 1 and 2, in Caspases activity assay there

is the opposite, even VUS-carriers 1 and 2 had statistical difference at 5µM of Doxorubicin. These differences can be related with the sensitivity of each technique and with the genetic variability, mentioned before. Because of that, it is essential to perform more experiments in each technique, knowing that we always take the risk that the donor may limit availability to the study by asking for more blood draws.

The γH2Ax assay has been used to assess Chemical and IR-induced damage in cells, once it is a based analysis marker for DNA double-strand breaks (DSBs) (Kataoka et al., 2006). When analysing the influence of Doxorubicin, in Figure 3.10, every samples showed statistically significance (p-value<0.05) in comparison between 5µM of Doxorubicin and negative control (NC - 0µM), and each one shows an increase of γH2Ax phosphorylated by increasing the concentration of Doxorubicin. However, it is showed that Controls 1 and 2 have lower values, compared with VUS-carriers 1 and 2, but due to the high SD observed for the highest dose it is assumed the existence of intra-individual variability. When comparing between samples (Figure 3.9), there is only statistical difference (p-value<0.05) between VUS-carrier 2 and Control 2 at 5µM of Doxorubicin, where the VUS-carrier 2 shows more damage (more γH2Ax phosphorylated) than Control 2. This is may indicate that Control 2 has less sensitivity to the drug than the VUS-carrier 2, which it may be related with a defective in DNA repair in this last one. However, there is intra-individual variability at least in Control 2, due the high SD, which does not clarify the correct results. Additionally, in this assay we should have used a positive control for quantify the DNA damage and the DSBs, like Ionizing radiation, which it is a type of high-energy radiation that is able to release electrons from atoms and molecules generating ions that can break covalent bonds. This type of radiation directly affects DNA structure and induces DNA breaks, particularly, DSBs (Borrego-Soto, Ortiz-López, & Rojas-Martínez, 2015). Once we do not used it, we do not know if the results that we obtained are related with the VUS or with the treatment, and because of that it is crucial make more assays, this time with a positive control that we know for sure that induce DSBs, to the correct understanding of the results.

Comet assay, like γH2Ax assay, has been used for quantifying and analysing DNA damage in individual cells (Fairbairn et al., 1995; Tice et al., 2000). When analysing Doxorubicin's influence (Figure 3.13) VUS-carrier 1 and Control 1 showed statistical significance (p-value<0.05) with negative control (NC - 0µM) at 5 and 10µM of Doxorubicin, with VUS-carrier 1 had higher values compared with Controls 1 and 2, even this last one did not show statistical difference between doses. However, for sample VUS-carrier 2 we should be expecting more damage after the treatment, just like VUS-carrier 1, but instead it shows no visible difference between concentrations, which can be explained by inter-individual variability and not directly related to the presence of VUS. When comparing between samples, in Figure 3.12, is much evident that at highest doses (5 and 10µM of Doxorubicin), VUS-carrier 1 and 2 shows statistical difference in comparison with Controls 1 and 2. However, this figure shows the inter-individual variability that exists between VUS-carriers and between Controls. Even that, VUS-carrier 1 shows more DNA in tail than Controls 1 and 2, which it may indicate that Controls have less sensitivity than the VUS-carrier 1 and that is possibly related with the defective in DNA repair. Additionally, our positive controls for DNA damage (10µM, 25µM and 50µM of H_2O_2) show statistical significance between some samples, showing that VUS-carrier 2 and Control 2 had similar results. In this case, inter-individual variability is observed again between VUS-carriers and between Controls. Because of these strange results, it is essential perform more assays to correct understanding the results.

When comparing the results of both techniques there is some differences. While in γH2Ax assay, all samples have statistical difference between 5µM of Doxorubicin and negative control (NC - 0µM), in Comet assay, only VUS-carrier 1 and Control 1 show that. Furthermore, instead of had statistical difference between samples at 5µM, like Comet assay has, in γH2Ax assay there is only one statistical difference and between VUS-carrier 2 and Control 2. Additionally, VUS-carrier 2 shows two different

behaviours, with the highest values for damage in the γH2Ax assay, while in Comet assay, the sample shows a behaviour similar to a negative control, almost without any difference between doses, even with the positive controls (10μM, 25μM and 50μM of H_2O_2). These differences may possible indicate inter- and intra-individual variabilities, like it was mentioned before, and maybe the existence of bias that we could not control, once each donor had to draw blood for several times for the several assays.

Even these differences, in both assays both Controls show less damage than VUS-carrier 1, and the damage increase in all these three samples by increasing the dose of Doxorubicin. This may indicate again that Controls had less sensitivity than VUS-carriers, and that sensitivity is possibly associated with a defective in DNA repair. However, more assays should be performed to assess the real picture of VUS-carrier 2 and confirm the results obtained.

In fact, our results were not conclusive concerning the biological role of VUS under study, and didn't let us state about its pathogenicity. Further studies shall be conducted that help us to confirm the clinical relevance of this VUS detected in *BRCA1* gene.

5. CONCLUSION

Over the years, breast cancer become the second most commonly diagnosed cancer worldwide and to overcome this problem genetic testing become essential for the identification of individuals and families with a genetic predisposition to developing cancer. With an increase of these tests, the appearing of VUS has become more common among the population. These variants cannot be classified as either benign or pathogenic, due to its inability to classify BRCA1/2 VUS and to determine which mutations are disease causing, which generates significant problems in risk evaluation, counselling and preventive care. Because of that are functional assays so important, once they work as independent classifiers of the VUS on their protein function, which can predict cancer causality of VUS in a likelihood model.

There are several functional assays, but in this study we only four of them: TUNEL assay, Caspases activity assay, γH2Ax assay and Comet assay. All of them showed some differences in all samples that may indicate the existence of intra- and inter-individual variability, due the fact that we used living cells (PBMCs) from the patients, which limit the cell supply. As expected, all experiments showed an increase of sensitivity by increasing the dose of the drug, and all of them, except TUNEL assay, showed a less sensitivity in Controls than the VUS-carrier 1 in drug response, which possibly be associated with a defective in DNA repair in VUS-carriers. VUS-carrier 2, on its turn, shows some results not expected, maybe because of genetic variability, even in positive controls. Because of that is not clear the real picture of VUS-carriers and if VUS has an important role in DNA repair. Concerning such fact, and even our results didn't allow us to hypothesize about the biologic risk of the VUS under study, left our mind aware to create new questions to explain the results here shown.

Thus, more assays should be performed to remove the genetic variability and to correlate all techniques in one answer that could us help to characterize the highly frequent VUS in cancer-related genes.

Due the fact that the biggest limitations of using Peripheral Blood Mononuclear Cells (PBMCs) are the recurrent blood collection by venous puncture from our patients, and the limited cell supply, mentioned before, make this a methodology invasive to the patients and to their families. Because of that we intend to establish human B lymphoblastic cell lines, derived from our patients, using Epstein-Barr virus, which it is going to overcome all of these problems. Furthermore, to help us on the characterization of highly frequent VUS in cancer-related genes detected by clinical panels through NGS platforms, we intend to use a breast cancer cell model (MCF-10A) that has been transformed through gene editing, CRIPSR-Cas9 approach, by introducing the VUS of interest in the cell-line genome. Then, functional assays will be performed exposing the cell model to different chemotherapeutic compounds such as: PARP inhibitors analogues, doxorubicin and platinum analogues, to evaluate their cytotoxic and genotoxic effects.

6. REFERENCES

Barnard, S., Ainsbury, E. A., Al-Hafidh, J., Hadjidekova, V., Hristova, R., Lindholm, C., … Rothkamm, K. (2015). The first gamma-H2AX biodosimetry intercomparison exercise of the developing european biodosimetry network RENEB. *Radiation Protection Dosimetry*, *164*(3), 265–270. https://doi.org/10.1093/rpd/ncu259

Blanpain, C. (2013). Tracing the cellular origin of cancer. *Nature Cell Biology*, *15*(2), 126–134. https://doi.org/10.1038/ncb2657

Bonassi, S., Znaor, A., Ceppi, M., Lando, C., Chang, W. P., Holland, N., … Fenech, M. (2007). An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis*, *28*(3), 625–631. https://doi.org/10.1093/carcin/bgl177

Borrego-Soto, G., Ortiz-López, R., & Rojas-Martínez, A. (2015). Ionizing radiation-induced DNA injury and damage detection in patients with breast cancer. *Genetics and Molecular Biology*, *38*(4), 420–432. https://doi.org/10.1590/S1415-475738420150019

Carvalho, M. A., Couch, F. J., & Monteiro, A. N. A. (2007). Functional assays for BRCA1 and BRCA2. *International Journal of Biochemistry and Cell Biology*, *39*(2), 298–310. https://doi.org/10.1016/j.biocel.2006.08.002

Copper, Geoffrey and Hausman, R. (2007). DNA Repair. In *Cell: A Molecular Approach* (2nd editio, pp. 650–654). Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK9900/

Daroui, P., Desai, S. D., Li, T. K., Liu, A. A., & Liu, L. F. (2004). Hydrogen Peroxide Induces Topoisomerase I-mediated DNA Damage and Cell Death. *Journal of Biological Chemistry*, *279*(15), 14587–14594. https://doi.org/10.1074/jbc.M311370200

de Jong, M., Nolte, I., te Meermanvan, G., van der Graaf, W., Oosterwijk, J., Kleibeuker, J., … de Vries, E. (2002). Genes other than BRCA1 and BRCA2 involved in breast cancer susceptibility. *Journal of Medical Genetics*, *39*(4), 225–242. https://doi.org/10.1136/jmg.39.4.225

Dinis, J., Silva, V., Gromicho, M., Martins, C., Laires, A., Tavares, P., … Rodrigues, A. (2012). DNA damage response in imatinib resistant chronic myeloid leukemia K562 cells. *Leukemia and Lymphoma*, *53*(10), 2004–2014. https://doi.org/10.3109/10428194.2012.681654

Fairbairn, D. W., Olive, P. L., & O'Neill, K. L. (1995). The comet assay: a comprehensive review. *Mutation Research/Reviews in Genetic Toxicology*, *339*(1), 37–59. https://doi.org/10.1016/0165-1110(94)00013-3

Feng, Y., Spezia, M., Huang, S., Yuan, C., Zeng, Z., Zhang, L., … Ren, G. (2018). Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. *Genes and Diseases*, *5*(2), 77–106. https://doi.org/10.1016/j.gendis.2018.05.001

Grogan, L., & Kirsch, I. R. (1997). Genetic testing for cancer risk assessment: A review. *Oncologist*, *2*(4), 208–222. https://doi.org/10.1634/theoncologist.2-4-208

Guidugli, L., Shimelis, H., Masica, D. L., Pankratz, V. S., Lipton, G. B., Singh, N., … Couch, F. J. (2018). Assessment of the Clinical Relevance of BRCA2 Missense Variants by Functional and Computational Approaches. *American Journal of Human Genetics*, *102*(2), 233–248. https://doi.org/10.1016/j.ajhg.2017.12.013

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, *144*(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Hassanpour, S. H., & Dehghani, M. (2017). Review of cancer from perspective of molecular. *Journal of Cancer Research and Practice*, *4*(4), 127–129. https://doi.org/10.1016/j.jcrpr.2017.07.001

Helena, J. M., Joubert, A. M., Grobbelaar, S., Nolte, E. M., Nel, M., Pepper, M. S., … Mercier, A. E. (2018). Deoxyribonucleic acid damage and repair: Capitalizing on our understanding of the mechanisms of maintaining genomic integrity for therapeutic purposes. *International Journal of Molecular Sciences*, *19*(4). https://doi.org/10.3390/ijms19041148

Hoeijmakers, J. H. J. (2009). *DNA Damage, Aging, and Cancer*. 1475–1485.

Hoffman-Andrews, L. (2017). The known unknown: The challenges of genetic variants of uncertain significance in clinical practice. *Journal of Law and the Biosciences*, *4*(3), 648–657. https://doi.org/10.1093/jlb/lsx038

Kamińska, M., Ciszewski, T., Łopacka-Szatan, K., Miotła, P., & Starosławska, E. (2015). Breast cancer risk factors. *Przeglad Menopauzalny*, *14*(3), 196–202. https://doi.org/10.5114/pm.2015.54346

Kataoka, Y., Bindokas, V. P., Duggan, R. C., Murley, J. S., & Grdina, D. J. (2006). Flow Cytometric Analysis of Phosphorylated Histone H2AX Following Exposure to Ionizing Radiation in Human Microvascular Endothelial Cells. *Journal of Radiation Research*, *47*(3/4), 245–257. https://doi.org/10.1269/jrr.0628

Kaufmann, S. H., Lee, S. H., Meng, X. W., Loegering, D. A., Kottke, T. J., Henzing, A. J., … Earnshaw, W. C. (2008). Apoptosis-associated caspase activation assays. *Methods*, *44*(3), 262–272. https://doi.org/10.1016/j.ymeth.2007.11.005

Kyrylkova, K., Kyryachenko, S., Leid, M., & Kioussi, C. (2012). Odontogenesis: Methods and Protocols. *Methods*

in Molecular Biology, *887*, 41–47. https://doi.org/10.1007/978-1-61779-860-3_5
Lam, F., & Ervik, M. (2018). Cancer Today - IARC. Retrieved from https://gco.iarc.fr/today/home
Li, P., Zhou, L., Zhao, T., Liu, X., Zhang, P., Liu, Y., … Li, Q. (2017). Caspase-9: Structure, mechanisms and clinical application. *Oncotarget*, *8*(14), 23996–24008. https://doi.org/10.18632/oncotarget.15098
Loo, D. T. (2011). DNA Damage Detection In Situ, Ex Vivo, and In Vivo: Methods and Protocols. *Methods in Molecular Biology*, *682*, 3–13. https://doi.org/10.1007/978-1-60327-409-8_1
Mahdavi, M., Nassiri, M., Kooshyar, M. M., Vakili-Azghandi, M., Avan, A., Sandry, R., … Gopalan, V. (2019). Hereditary breast cancer; Genetic penetrance and current status with BRCA. *Journal of Cellular Physiology*, *234*(5), 5741–5750. https://doi.org/10.1002/jcp.27464
Majidinia, M., & Yousefi, B. (2017). DNA repair and damage pathways in breast cancer development and therapy. *DNA Repair*, *54*(March), 22–29. https://doi.org/10.1016/j.dnarep.2017.03.009
Miller-Samuel, S., McDonald, D. J., Weitzel, J. N., Santiago, F., Martino, M. A., Namey, T., … Morris, G. J. (2011). Variants of uncertain significance in breast cancer-related genes: Real-world implications for a clinical conundrum. Part one: Clinical genetics recommendations. *Seminars in Oncology*, *38*(4), 469–480. https://doi.org/10.1053/j.seminoncol.2011.04.008
Moghadasi, S., Eccles, D. M., Devilee, P., Vreeswijk, M. P. G., & van Asperen, C. J. (2016). Classification and Clinical Management of Variants of Uncertain Significance in High Penetrance Cancer Predisposition Genes. *Human Mutation*, *37*(4), 331–336. https://doi.org/10.1002/humu.22956
Nagahashi, M., Shimada, Y., Ichikawa, H., Kameyama, H., Takabe, K., Okuda, S., & Wakai, T. (2019). Next generation sequencing-based gene panel tests for the management of solid tumors. *Cancer Science*, *110*(1), 6–15. https://doi.org/10.1111/cas.13837
Nowsheen, S., & Yang, E. S. (2013). the Intersection Between Dna Damage Response and. *Experimental Oncology*, *34*(3), 243–254.
Papaliagkas, V., Anogianaki, A., Anogianakis, G., & Ilonidis, G. (2007). The proteins and the mechanisms of apoptosis: A mini-review of the fundamentals. *Hippokratia*, *11*(3), 108–113.
Psyrri, A., Apostolou, P., Fostira, F., Economopoulou, P., & Dimitriadis, G. (2015). Genetics Home Reference Breast Cancer. *U.S. National Library of Medicine*, 1–9. Retrieved from https://ghr.nlm.nih.gov/condition/breast-cancer
Roos, W. P., & Kaina, B. (2006). DNA damage-induced cell death by apoptosis. *Trends in Molecular Medicine*, *12*(9), 440–450. https://doi.org/10.1016/j.molmed.2006.07.007
Shiovitz, S., & Korde, L. A. (2015). Genetics of breast cancer: A topic in evolution. *Annals of Oncology*, *26*(7), 1291–1299. https://doi.org/10.1093/annonc/mdv022
Swift, L. H., & Golsteyn, R. M. (2014). Genotoxic anti-cancer agents and their relationship to DNA damage, mitosis, and checkpoint adaptation in proliferating cancer cells. *International Journal of Molecular Sciences*, *15*(3), 3403–3431. https://doi.org/10.3390/ijms15033403
Tice, R. R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., … Sasaki, Y. F. (2000). Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis*, *35*(3), 206–221. https://doi.org/10.1002/(SICI)1098-2280(2000)35:3<206::AID-EM8>3.0.CO;2-J
Toland, A. E., & Andreassen, P. R. (2017). DNA repair-related functional assays for the classification of BRCA1 and BRCA2 variants: A critical review and needs assessment. *Journal of Medical Genetics*, *54*(11), 721–731. https://doi.org/10.1136/jmedgenet-2017-104707
Tram, E., Savas, S., & Ozcelik, H. (2013). Missense Variants of Uncertain Significance (VUS) Altering the Phosphorylation Patterns of BRCA1 and BRCA2. *PLoS ONE*, *8*(5). https://doi.org/10.1371/journal.pone.0062468
Ward, T. H., Cummings, J., Dean, E., Greystoke, A., Hou, J. M., Backen, A., … Dive, C. (2008). Biomarkers of apoptosis. *British Journal of Cancer*, *99*(6), 841–846. https://doi.org/10.1038/sj.bjc.6604519
Weeden, C. E., & Asselin-Labat, M. L. (2018). Mechanisms of DNA damage repair in adult stem cells and implications for cancer formation. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1864*(1), 89–101. https://doi.org/10.1016/j.bbadis.2017.10.015
Whitney, A. R., Diehn, M., Popper, S. J., Alizadeh, A. A., Boldrick, J. C., Relman, D. A., & Brown, P. O. (2003). Individuality and variation in gene expression patterns in human blood. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(4), 1896–1901. https://doi.org/10.1073/pnas.252784499
WHO. (2014). WHO | Cancer. *WHO Factsheet 297*, p. 1. Retrieved from http://www.who.int/mediacentre/factsheets/fs297/en/
Woods, D., & Turchi, J. J. (2013). Chemotherapy induced DNA damage response Convergence of drugs and pathways. *Cancer Biology and Therapy*, *14*(5), 379–389. https://doi.org/10.4161/cbt.23761
Wu, C. C., & Bratton, S. B. (2017). Caspase-9 swings both ways in the apoptosome. *Molecular and Cellular Oncology*, *4*(2), 1–3. https://doi.org/10.1080/23723556.2017.1281865

Yurtcu, E., Işeri, D., & Sahin, F. I. (2014). Genotoxic and cytotoxic effects of doxorubicin and silymarin on human hepatocellular carcinoma cells. *Human and Experimental Toxicology*, *33*(12), 1269–1276. https://doi.org/10.1177/0960327114529453

Zeng, C. W., Zhang, X. J., Lin, K. Y., Ye, H., Feng, S. Y., Zhang, H., & Chen, Y. Q. (2012). Camptothecin induces apoptosis in cancer cells via microRNA-125b-mediated mitochondrial pathways. *Molecular Pharmacology*, *81*(4), 578–586. https://doi.org/10.1124/mol.111.076794

7. APPENDIX

Exma. Senhora,

O estudo que pretendemos desenvolver será realizado no Departamento de Genética da Faculdade de Ciências Médicas da Universidade Nova de Lisboa e tem como título "Cancro da Mama: a relevância clínica de variantes de significado desconhecido (VUS) em genes de reparação por recombinação homóloga – análise funcional em doentes com cancro de mama familiar". Pretende-se estudar a relevância clínica de alterações em genes associados com o aumento de risco para o cancro de mama familiar.

Este estudo está inserido numa bolsa financiada pela Liga Portuguesa Contra o Cancro (LPCC-NRS) – Terry Fox 2017 - sob responsabilidade da Doutora Susana Silva, investigadora da NMS|FCM - UNL em parceria com a Ophiomics na pessoa do Doutor José Leal, e ainda com a Doutora Octávia Monteiro Gil (CTN-IST-UL). O estudo consistirá numa avaliação da resposta celular a exposições genotóxicas induzidas por agentes lesivos para o ADN.

Durante este estudo será recolhida uma amostra de sangue total a partir da qual se realizarão todos os ensaios *in-vitro* associados ao estudo. Será necessário recolher alguma informação pessoal que será tratada de forma confidencial e anónima. Este estudo não acarreta benefícios diretos para si, nem quaisquer riscos. É garantida a total confidencialidade e anonimato dos dados colhidos, nomeadamente a sua identificação só será conhecida pelo investigador responsável e não estará associada diretamente à manipulação da amostra.

Este estudo será realizado sempre com o acompanhamento do investigador responsável e colaboradores que manipularão amostras já codificadas e totalmente anónimas.

Agradecendo antecipadamente a sua colaboração,

Doutora Susana Nunes da Silva
Investigadora / Professora Auxiliar Convidada
Faculdade de Ciências Médicas da UNL

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Figure 7.1 - Study's information delivered to the donors.

ID AMOSTRA: _____

DECLARAÇÃO

Eu,_____, declaro ter sido devidamente esclarecida sobre a natureza e objetivos do estudo *"Cancro da Mama: a relevância clínica de variantes de significado desconhecido (VUS) em genes de reparação por recombinação homóloga – análise funcional em doentes com cancro de mama familiar"*, tendo decidido colaborar voluntariamente neste estudo.

Assinatura do Participante

_____

Assinatura Investigador responsável

_____

Lisboa, ____ de _____ de ____

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Figure 7.2 - Informed consent filled and signed by donors.

ID AMOSTRA: _____

INQUÉRITO

Género: Feminino ☐ Masculino ☐

Data de Nascimento: _____

Gestações: _____ **Amamentação:**_____

Fumador: Sim ☐ Não ☐ Ex-Fumador ☐ _____

Consumo de Álcool: Regular/Refeições ☐ Social ☐ Nunca☐

Restrições Alimentares: Não ☐ Sim ☐ Quais:_____

Data Diagnóstico Patologia Oncológica: _____

Antecedentes Familiares doença oncológica: _____

Outras patologias: _____

Toma medicação regularmente: Não ☐ Sim ☐ Qual:_____

Métodos Complementares de Diagnóstico: _____

Data dos últimos exames radiológicos (e.g. Raios-X, ortopantomografias):

_____

Notas Relevantes: _____

_____

_____

_____

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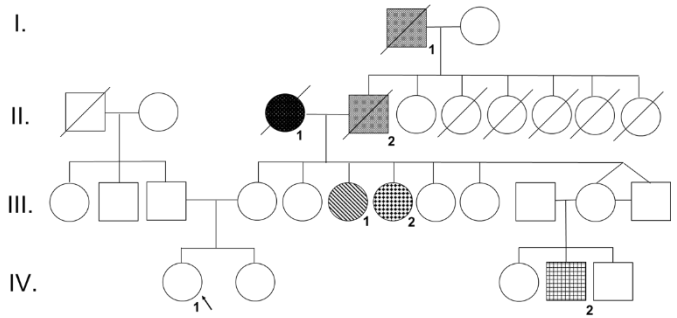
Figure 7.3 - Questionnaire filled and signed by donors.

Table 7.1 - Results obtained from sequencing with the alteration present in each donor and probable outcomes predicted.

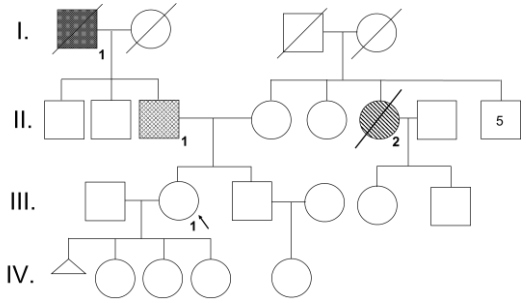
ID	Age	Cancer	Family History	Gene	VUS	EBI amino/genomic	rs	Cons. Type	PolyPhen	Clin. Signif.	Diagnosis
VUS-carrier 1	25	Healthy	Yes	BRCA1	NM_007294.3:c.1067A>G NM_007297.3:c.926A>G	ENSP00000350283.3:p.Gln356Arg 17:g.43094464T>C	rs1799950	Missense	probably damaging(0.969)	Benign	Benign
VUS-carrier 2	39	Healthy	Yes	BRCA1	NM_007294.3:c.1067A>G	ENSP00000418960.2:p.Gln356Arg 17:g.43094464T>C	rs1799950	Missense	possibly damaging(0.795)	Benign	Benign

VUS Carrier 1



I.1. Prostate Cancer; ?
II.1. Esophageal Cancer; 75y
II.2. Prostate Cancer; 80y
III.1. Hypophysis Adenoma; 50y
III.2. Tumoral Mass Intraperitoneal; 65y
IV.1. Healthy; Mutation Carrier; 25y
IV.2. Thyroid Carcinoma; 20y

VUS Carrier 2



I.1. Unknown Cancer; ?
II.1. Chronic Thrombocytopenia; 69y
II.2. Ovarian Cancer; >50<60y
III.1. Healthy; Mutation Carrier; 39y

Figure 7.4 - Pedigree Diagrams and family history of samples VUS-carrier 1 and VUS-carrier 2.

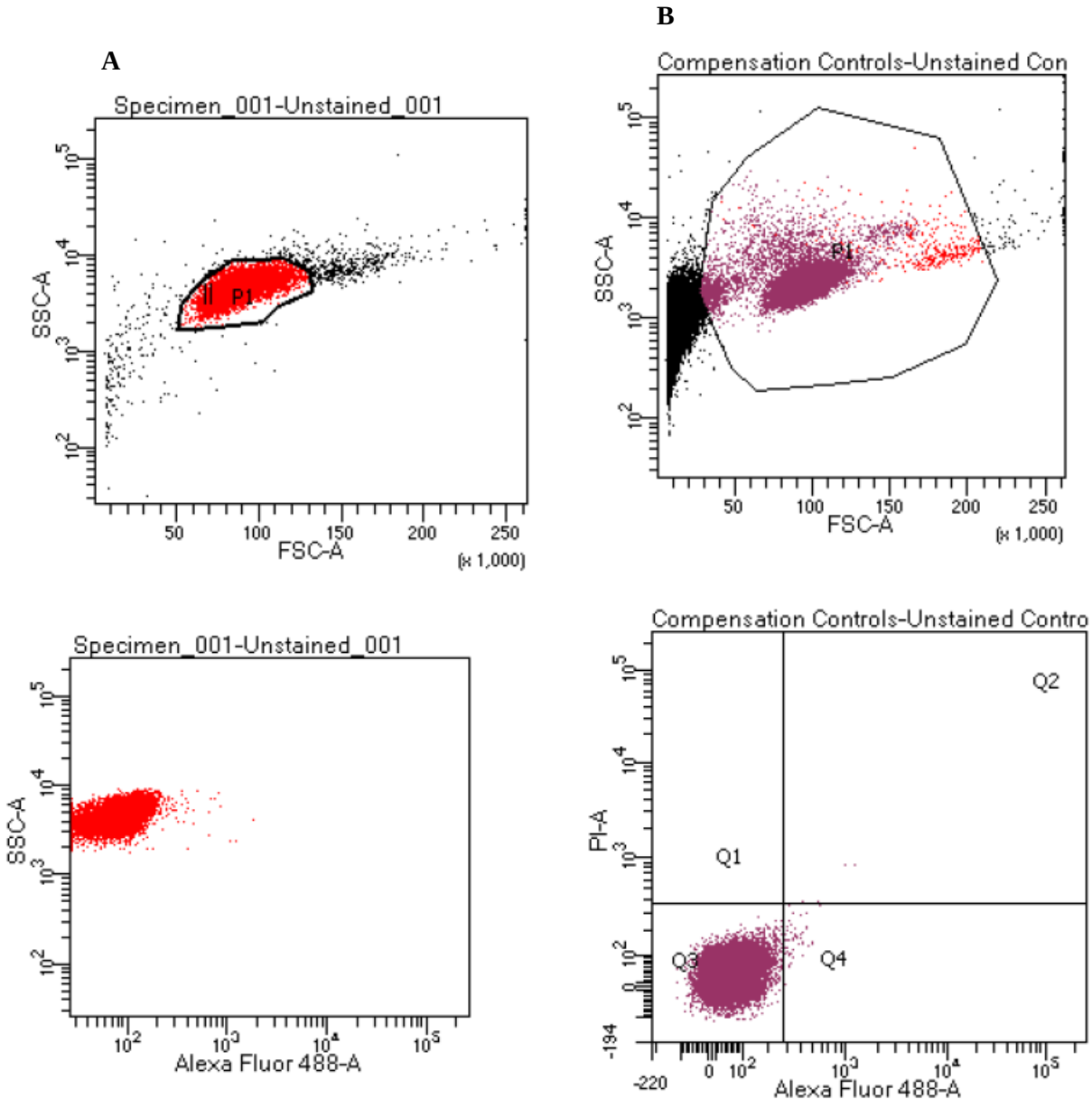


Figure 7.5 - The experimental batch of sample preparation using PBMCs. (A) TUNEL assay; (B) Caspases activity assay.

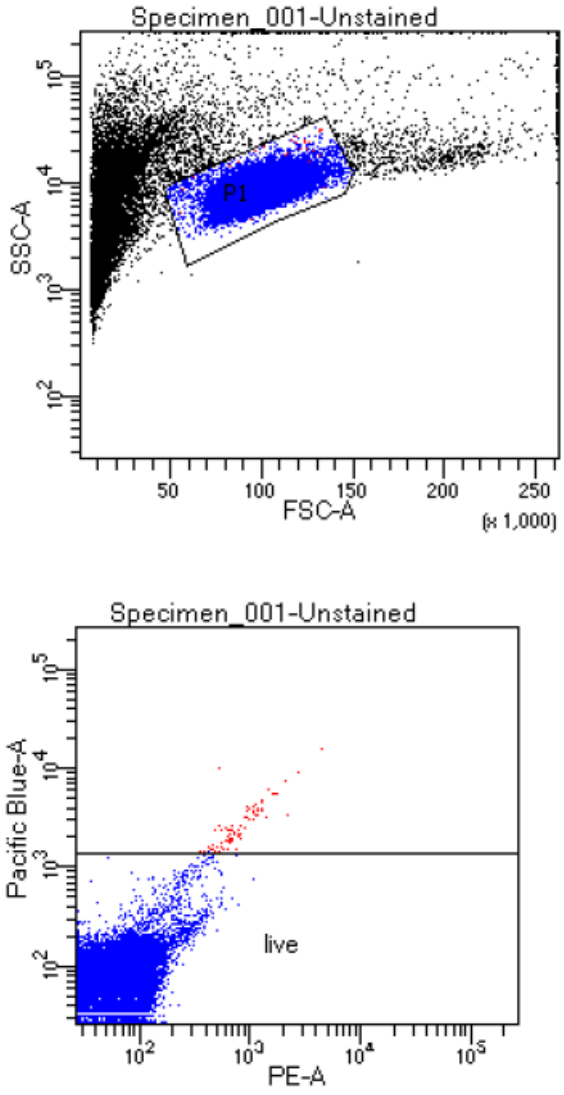


Figure 7.6 - The experimental batch of sample preparation for the γH2AX assay using PBMCs.